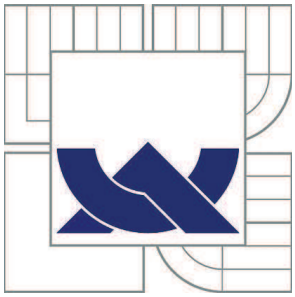

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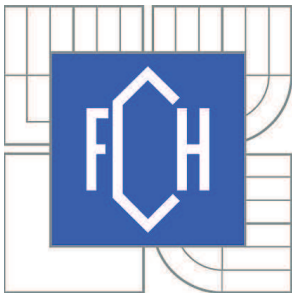

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ÚSTAV CHEMIE POTRAVIN A BIOTECHNOLOGIÍ

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# ANTIMICROBIAL ACTIVITY OF CARBON-BASED FILLERS

ANTIMIKROBIÁLNÍ AKTIVITA UHLÍKATÉHO PLNIVA

DIPLOMOVÁ PRÁCE

MASTER'S THESIS

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## ABSTRACT

The aim of this diploma thesis is focused on the impact of carbon-based fillers on viability and extracellular substances production by bacterium *Bacillus subtilis* (CCM 1999) and yeast *Yarrowia lipolytica* (CCY 29-26-52). Antimicrobial activity of these particles, present in cultivation nutrient medium was examined using following parameters: growth of mentioned microorganisms, production of extracellular proteins and finally extracellular polymeric substances production, which is strongly connected with biofilm formation.

Nanomaterials suspension (0.135 mg/mL) was prepared in two different cultivation media i.e. nutrient medium supplemented with glucose for *Bacillus subtilis* and basal medium with the addition of 2% (vol.) Tween 80 for *Yarrowia lipolytica* and media were inoculated by appropriate type of microorganism. Experiments were performed for 6 days under shaking rate at 160 rpm and at temperature of 30 °C for *Bacillus subtilis* and 28 °C for *Yarrowia lipolytica*.

Three types of carbon nanomaterials obtained from Department of Inorganic Chemistry, Institute of Chemical Technology, Prague were examined. These materials specified as material "A", "B" and "C" are mutually different by the size of its particles and the degree of oxidation.

Based on the screening studies the tested material concentration of 0.135 mg/mL and shaking rate of 160 rpm were chosen.

According to the optical density measurement at 600 nm, the growth curves of both microorganisms in the presence of tested nanoparticles during 5 days period were compared. It was find out, that the presence of nanoparticles don't have a significant influence on tested microorganisms growth, by this technique. However, this method is just wider point of view, due to mistakes caused by presence of dead cells.

Further, production of total cells proteins and extracellular proteins by microorganisms in presence of tested nanoparticles was examined. There was not observed any significant deviation from control samples values, where the tested materials were absent.

Based on colony counting method (used for *Bacillus subtilis*) and cells counting in Bürker counting chamber (used for *Yarrowia lipolytica*), loss of microorganism viability was determined in 3 cultivation periods (6, 48 and 144 hours); there was observed a support of growth of microorganisms rather in shorter incubation period.

Thereafter the extracellular polymeric substances **(**EPS) production that means proteins, reducing substances and polysaccharides was monitored. These substances were secreted into the medium by mentioned microorganisms during 24 hours of incubation. *Bacillus subtilis* cells produce much more EPS than *Yarrowia lipolytica* cells. We suppose that the EPS production could be closely associated with production of biofilm, which protects cells against nanoparticles toxicity.



## KEYWORDS

Carbon-based nanomaterials, antimicrobial activity, extracellular polymeric substances, *Bacillus subtilis*, *Yarrowia lipolytica*

## ABSTRAKT

Diplomová práce se zabývá vlivem uhlíkatého plniva na životaschopnost a produkci extracelulárních látek vybrané bakterie *Bacillus subtilis* (CCM 1999) a kvasinky *Yarrowia lipolytica* (CCY 29-26-52). Antimikrobiální aktivita těchto částic, přítomných v kultivačním mediu, byla sledována pomocí následujících parametrů: růst daného mikroorganismu, produkce extracelulárních proteinů a v poslední řadě byla monitorována produkce extracelulárních polymerních substancí, které mají úzkou souvislost s tvorbou biofilmu.

Suspenze materiálů (0,135 mg/mL) byly připraveny ve dvou rozdílných kultivačních mediích; tzn. živné medium s obsahem glukózy pro *Bacillus subtilis* a bazální medium s přídavkem Tweenu 80 pro *Yarrowia lipolytica*, a media byla inokulována příslušným typem mikroorganismu. Experimenty probíhaly po dobu 6 dnů při rychlosti třepání 160 rpm a teplotě 30 °C pro *Bacillus subtilis* a 28 °C pro *Yarrowia lipolytica*.

Testovány byly celkem tři typy uhlíkatého nanomateriálu, získané z Katedry anorganické chemie, Vysoké školy chemicko-technologické v Praze. Tyto materiály specifikované jako materiál "A", "B" a "C" se navzájem lišily velikostí částic a stupněm oxidace.

Na základě skríningových studií byla vybrána koncentrace testovaného materiálu 0,135 mg/mL a rychlost třepání 160 rpm.

Metodou měření optické hustoty vzorku při 600 nm byly sestaveny a porovnány růstové křivky obou mikroorganismů v přítomnosti testovaných nanočástic po dobu 5 dní. Tímto způsobem bylo zjištěno, že přítomnost nanočástic v mediu nemá velký vliv na růst zkoumaného mikroorganismu. Tato metoda, je však pouze orientační, protože se nevyhneme chybě díky přítomnosti mrtvých buněk.

Dále byla testována produkce celkových a extracelulárních proteinů daným mikroorganismem v přítomnosti testovaných nanočástic. Nebyla však pozorována výrazná odchylka hodnot od hodnot kontrolního vzorku, který neobsahoval testovaný materiál.

Na základě metod počítání kolonií (*Bacillus subtilis*) a buněk (*Yarrowia lipolytica*) byly určeny ztráty životaschopnosti mikroorganismu ve 3 časech (6, 48 a 144 hodin); v kratším časovém intervalu byl růst spíše podporován.

Dále byla monitorována produkce extracelulárních polymerních substancí (EPS), tedy proteinů, redukujících substancí a polysacharidů. Tyto látky byly vylučovány daným mikroorganismem do prostředí v průběhu 24 hodin. *Bacillus subtilis* produkoval EPS ve větší míře než *Yarrowia lipolytica*. Předpokládáme, že produkce EPS by mohla souviset s tvorbou biofilmu, který chrání buňky před toxicitou nanočástic.



## KLÍČOVÁ SLOVA

Nanomateriály na bázi uhlíku, antimikrobiální aktivita, extracelulární polymerní substance, *Bacillus subtilis*, *Yarrowia lipolytica*

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## DECLARATION

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……
student's signature

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# 1 INTRODUCTION

There was a huge progress in the development of nanotechnology during the first decade of 21th century. This development was precisely aimed for synthesis, characterization and application of new materials with at least one dimension till 100 nm [1].

Together with the discovery of these new particles, it should be considered the research of their harmlessness for human and basically the whole living world. The biological safety induces more and more attention from the government point of view and the scientists as well [2].

Actual nanosafety research is aimed to a control of development of small sizes materials, which can be industrially produced in large quantities and therefor there is a possibility of exposure of people or natural environment to these materials [3].

During the last thirty years, zero-dimensional, one-dimensional, and two-dimensional carbon nanomaterials (i.e., fullerenes, carbon nanotubes, and graphene) have been considered as high interesting, due to their excellent physiochemical and biological properties [4].

These nanomaterials due to their unique properties have been already applied in many fields of human life; such are energy storage, electrochemical devices, adsorption of enzymes, biosensors, cell imaging, drug delivery and as the filler materials for biomedical applications. One of the most attractive properties of nanomaterials is their antimicrobial activity [2, 5, 6].

There is a really intensive safety research of the impact of these particles on mammalian cells *in vitro* and if the graphene materials have the potential to induce foreign body sarcomas [3].

The research of antimicrobial activity of graphene based materials is another important sector of examination. It was issued a long row of contradictory science articles about the biocompatibility and antimicrobial activity of graphene materials. Liu *at al.* say, that graphene has strong cytotoxicity toward bacteria [7]. On the other hand Ruiz *at al.* published their results which show that in presence of graphene materials bacterial cultures grow faster and to a higher optical density than without tested material [8].

The aim of this work is examine antimicrobial activity or possible biocompatibility of three chosen carbon-based nanomaterials to a viability of model microorganisms such as bacterium (*Bacillus subtilis*) and yeast (*Yarrowia lipolypica*), due to this contradictory information.

These microorganisms mentioned above were cultivated in the presence of tested materials during different time periods. The aim of these tests was to examine their viability and production of extracellular polymeric substances (EPS) and proteins in given time intervals. EPS are essential for biofilm formation and protein production indicates the status of metabolism, which is exposed to the action of mentioned carbon nanoparticles. I will try to determine in this thesis, if one of the reactions of these microorganisms in presence of graphene material is biofilm creation and increasing of EPS production; and next how the microorganisms behave in presence of these particles and how react.

# 2 THEORETICAL PART

## 2.1 CARBON NANOMATERIALS

During the last thirty years, zero-dimensional, one-dimensional, and two-dimensional carbon nanomaterials (i.e., fullerenes, carbon nanotubes, and graphene) have been considered as high interesting, due to their excellent physiochemical and biological properties and diverse applications [4].

Graphene is a single-atom-thick, two-dimensional sheet of sp2-hydridized hexagonally arranged carbon atoms, which was isolated from crystalline graphite. Characterization of this unique material led to the Nobel Prize in 2010 [9].

Attractiveness of this material is due to a wide range of unusual properties, such as its large specific surface area, unusual structural characteristics, superlative mechanical strength, high optical transmittance, remarkable electronic properties and high intrinsic mobility [10–13].

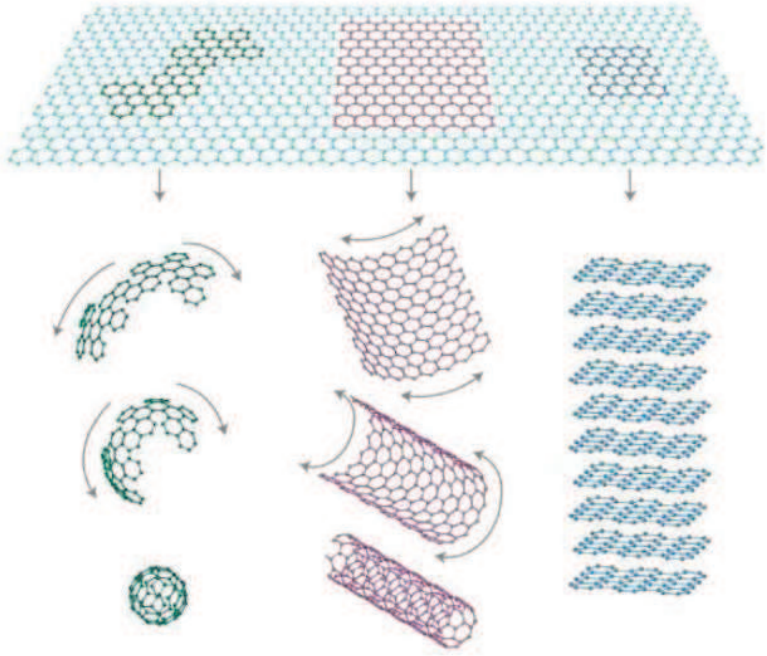

*Fig. 1 Schema of structure of carbon nanomaterials [14]*

There are plenty of variations of graphene materials. The differences are in layer number, lateral dimension, surface chemistry, defect density or quality of the individual graphene sheets, and composition or purity. Considering this, graphene-family nanomaterials (GFNs) are analogous to carbon nanotubes, which could vary in wall number, diameter, length, surface chemistry and the amount, composition, and physical form of metal impurities [3]. There are four types of graphene-based materials including graphene oxide (GO), reduced graphene oxide (rGO), graphite (Gt) and graphite oxide (GtO), which are very interesting and widely studied for their unique properties [7].

There are various methods how to prepare graphene. The most important are micromechanical exfoliation and chemical vapor deposition (CVD) method.

Micromechanical exfoliation is the longest used method to prepare the sheet of graphene. The elementary procedure for the exfoliation is repeated peeling. The bond between the layers of graphene sheet of graphite was break using the mechanical energy during the exfoliation [9].

An alternative method how to produce graphene is CVD method due to the graphene yield is too low by micromechanical exfoliation method. Transition metals are usually used as catalysts for the CVD process and hydrocarbon gases were used as precursor [15].

One of the types of carbon-based nanomaterial are carbon nanotubes (CNTs) with structure of one or more layers of graphene sheets for single walled carbon nanotubes (SWCNTs) or multi walled carbon nanotubes MWCNTs [16]. SWCNTs are the simplest forms of CNTs and are fully defined by their chiral angle, band gap and diameter. Usually, SWCNTs are divided into three basic different types: metallic, semimetallic, and semiconductive, depending on the rolling action of graphene sheet [15].

For our work, the most important graphene oxide (GO) is a graphene sheet with carboxylic groups at its edges and phenol hydroxyl and epoxide groups on its basal plane [7, 17, 18]. There are several possibilities how to prepare GO. One of the options is to exfoliate chemically GO from graphite oxide (GtO) [17]. For production of reduced graphene oxide (rGO) the chemical treatment or thermal annealing is possible to use, because these methods can eliminate functional groups on GO [19].

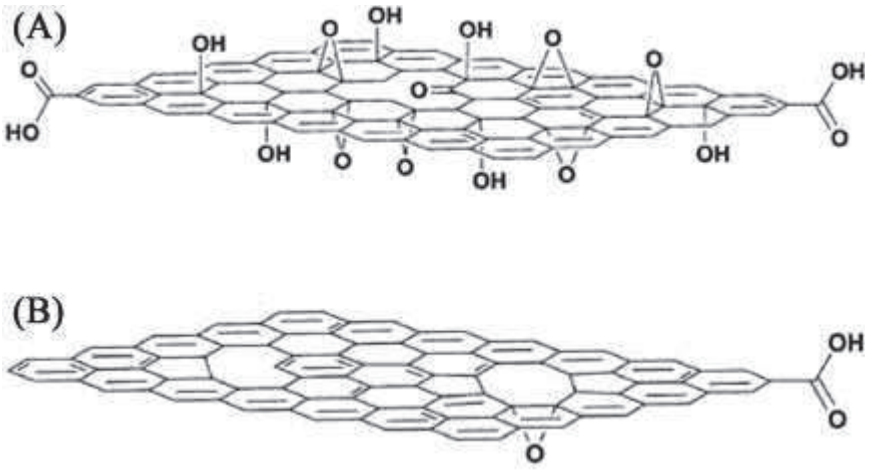


*Fig. 2 Schematic structure of a GO sheet (A) and rGO sheet (B) [18]*

## 2.2 THE PROMISING MATERIAL - GRAPHENE - OXIDE

Graphene oxide (GO) is possible to describe like a highly oxidized form of chemically modified graphene, which is produced by harsh oxidation of crystalline graphite followed by sonication or other dispersion methods to produce monolayer material, typically in aqueous suspension [3, 20]. GO samples could include not only monolayers but also multilayer flakes in their structure [3].

Reduced graphene oxide (rGO) is the product of treating GO under reducing conditions, that means high-temperature thermal treatment and chemical treatments with hydrazine ($N_2H_4$) or using other reducing agents [20]. The aim of GO reduction is often done to restore electrical conductivity, and it modifies many other GO properties and abilities as well. It reduces oxygen content, increases hydrophobicity, introduces holes or defects in the carbon lattice due to $CO/CO_2$ liberation. It also reduces surface charge and water dispersibility [21, 3].

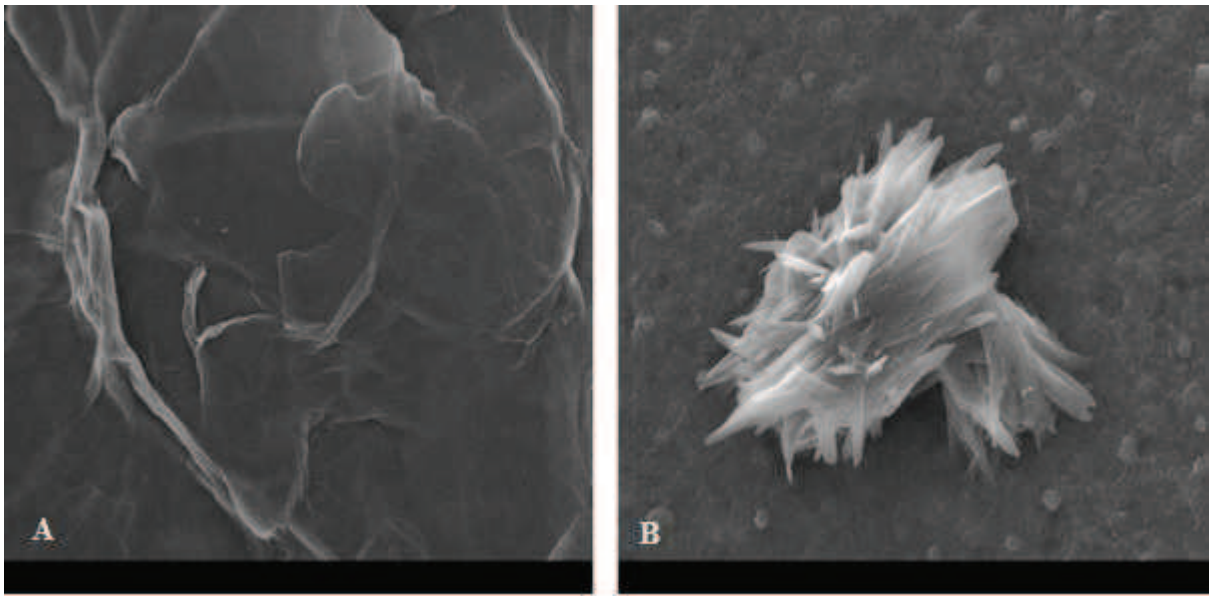


*Fig. 3 Scanning electron microscopy images of GO (A) and rGO (B) [22]*

The properties of graphene-family nanomaterials (GFNs) most relevant for their biological effects include not just surface area, but also layer number, lateral dimension, surface chemistry, and purity. Surfaces area plays a main role in the biological interactions of nanomaterials [23].

Because of the high surface area of GFNs, especially monolayer graphene and GO, we anticipate that surface phenomena, either physical adsorption or catalytic chemical reaction, will be of really high importance in the biological reaction to these materials [24, 25].

The amount of graphene layers in a GFN is important because it determines specific surface area and bending stiffness. It is expected that the adsorptive capacity for biological molecules will increase considerably as layer amount decrease [3].

The graphene family contains materials with broadly varying surface chemistry, even before any specific biofunctionalization is carried out. Graphene oxide surfaces are partially hydrophobic with hydrophilic (typical water contact angle is of 40–50°) regions [3, 26, 27] capable of hydrogen bonding and metal ion complexing [3, 28] and contain negative charges on edge sites associated with carboxylate groups [3, 29].

## 2.3 TOXICITY OF CARBON NANOMATERIALS

Due to rapid expansion of carbon nanomaterials in research and industries growing need for appropriate toxicological investigation in details.

There are four different entry ways for entering nanomaterials into the human body: inhalation, ingestion, dermal penetration, and injection or implantation for biomedical applications [3]. For many materials, inhalation exposure often contributes the highest risk, and there is significant evidence that correlates inhaled ultrafine and ambient particles with negative health effects [3, 30–32].

Some GFNs are prepared as dry powders for which inhalation exposures must at least be took as a possibility. GFNs are unique powders with plate-like structure, atomic thinness, and extreme aspect ratio [3].

Inhaled particles may deposit in numerous regions of the respiratory tract by impaction, sedimentation, and diffusion, or they may be exhaled. The particles retention time depends on their site of deposition and interactions with the airway surface. If the particles deposit in the conducting airways, the time of retention is short, because of the efficient mucociliary and cough clearance. The key role of the conducting airways is to act as a conduit for movement of air into the respiratory tract and to filter, warm, and humidify the incoming air. Mucociliary clearance is the predominant mechanism of particle clearance in the conducting airways. The rate of clearance depends on ciliary function and physical parameters of the respiratory tract lining fluids. As particles deposit more proximally, deeper into the lung, the retention time increases as a result of decreasing mucociliary clearance [3].

In several in vivo studies the chronic toxicity associated with GO was proved. GO was chiefly deposited in the lung after intravenous injections and caused pulmonary edema and lung granuloma creation [1, 33, 34].

Between inhalable particles is possible to find those having a regular geometric shape, while others such as agglomerates or crushed materials have irregular shapes. The shape of a particle impacts its drag force and settling speed; thus, a correction factor, a shape factor, is applied to account for the effect of shape on particle motion. It should be mentioned, that some GFNs are atomically thin, and all GFNs have very high aspect ratios that deviate markedly from spherical or equi-axed particles [3].

Finally, Sanchez *et al*. note that GFNs in the dry state, like other nanomaterials, have a strong affinity to aggregate into stacked plate structures and sometimes may also fold or crumple during processing and that each of these behaviors will modify their effective shape and deposition patterns [3].

And conclusion, Sanchez *et al*. note that it is important to study the interactions between GFNs and respiratory tract lining fluids in order to understand natural protective defense mechanisms. Alternatively, hydrophobic GFNs that enlarge mucin pores may increase sensibility to microbial penetration and infection [3, 35].

After penetration of the nanoparticles in the human body with all the above described ways, the main toxicity mechanism has been hypothesized, such as oxidation stress and disruption of membrane damage [15].

Sanchez *et al*. focused on exploring toxicity for mammalian cell in vitro and on potential for foreign body tumorigenesis.

**Mammalian cell toxicity *in vitro*:** Macrophages are the initial cells that respond to inhaled microorganisms or particulates [36]. The interaction between dispersed graphene or GO sheets and target cells has been studied in monolayer cultures of lung epithelial cells [37], fibroblasts [33], and neuronal cells [38]. Zhang *et al*. [38] described that few-layer graphene enlarged intracellular creation of reaction oxygen species (ROS) and induced mitochondrial damage in neuronal cells at a dose of 10 μg/mL after exposition during 4 and 24 hours. Surface modification of graphene materials have been reported to modify its toxicity [39]. And moreover it has been reported that rGO and carboxylated graphene are less toxic than GO or native graphene [40].

**Potential for Foreign Body Tumorigenesis:** Implantable sensors, tissue scaffolds, or coatings on prosthetics or implanted devices could be considered as future biomedical applications of graphene-family materials. Non-biodegradable foreign materials have induced sarcomas in rodents following implantation at a variety of anatomic locations (reviewed in IARC) [41]. This phenomenon is called solid state or foreign body carcinogenesis, and it is theorized to be induced by biopersistent, smooth, continuous surfaces irrespective of chemical composition [3, 42–44].

It is unknown whether graphene-family materials have the potential to induce foreign body sarcomas. The biomaterial properties related with foreign body sarcomas, however, include large size or surface area, smooth continuous surface, and biopersistence [3, 45]. Rough surfaces, powdered materials, nonmetallic particulates, and porous materials are less likely to induce tumors [3, 41]. GFNs can have very high surface areas, smooth topography, and may be biopersistent similar to tumorigenic solid-state implants [3, 46].

Several mechanisms have been considered for foreign body tumorigenesis. Direct physical contact between progenitor or preneoplastic cells with the surface of a smooth, contiguous, biopersistent implant has been hypothesized as essential for carcinogenicity [3, 42]. It is unlikely that additives, chemicals, or metal ions that leach slowly from medical implants contribute to carcinogenicity because inert biomaterials such as aluminum oxide ceramic and stable polymers including polytetrafluoroethylene produce foreign body sarcomas [3, 41].

## 2.4 ANTIMICROBIAL ACTIVITY OF CARBON NANOMATERIALS

Graphene-based materials need to be carefully evaluated before potential application due to the health and environmental impacts.

Liu *et al*. reported in their review that graphene has strong cytotoxicity toward bacteria generally and studied this problematic on four types of graphene-based materials (Gt, GtO, GO and rGO). *E. coli* was used as a model bacterium in this experiment. The death rate of bacterial cells was determined by the colony counting method and the isotonic saline solution without graphene-based materials was used as a control. From this experiment were created a few findings: the GtO dispersion shows a slight weaker antibacterial activity in comparison with Gt; GO have a much stronger bacterial activity in comparison with GtO and rGO has a

lower antibacterial activity compared with GO. In particular, GO and rGO have much higher bacterial inactivation percentages compared with those of Gt and GtO [7].

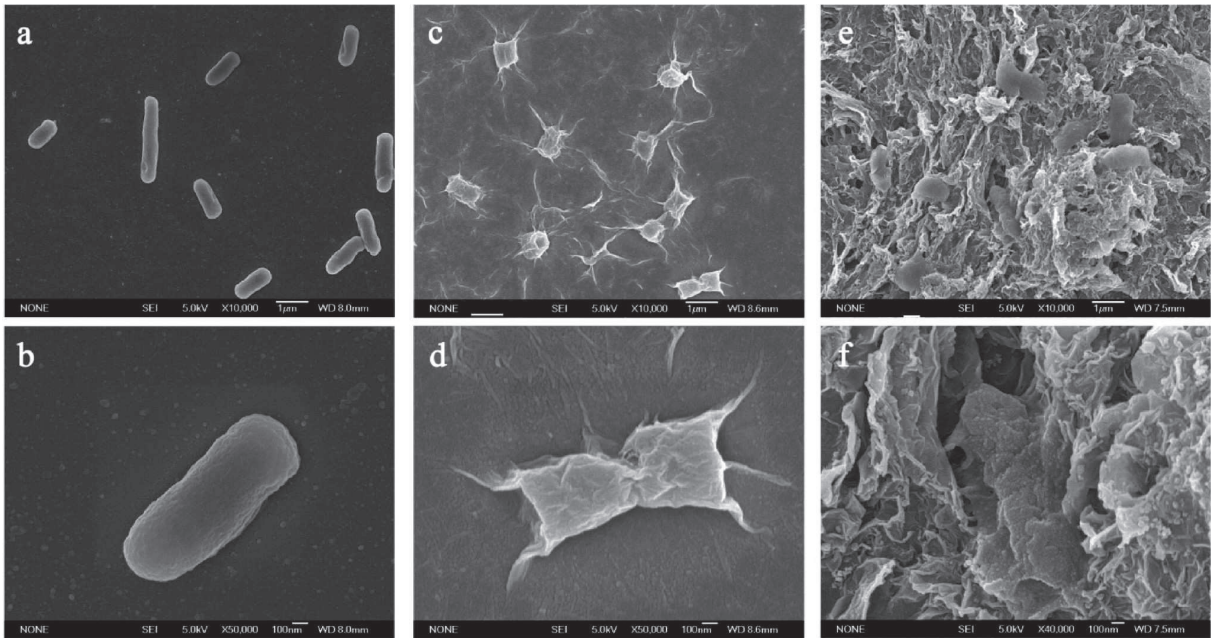


*Fig. 4 SEM images after 2 hours of incubation: E. coli after incubation with saline solution without graphene-based materials (a, b); E. coli cells after incubation with GO dispersion (40 μg/mL) (c, d); E. coli cells after incubation with rGO dispersion (40 μg/mL) (e, f) [7]*

Studies with the same materials but with a different microorganism also performed Gurunathan *et al*. They studied the growth curve of *P. aeruginosa* under aerobic conditions with and without Gt, GtO, GO, and rGO tested materials in concentration of 75 μg/mL for 15 hours. Results of this study are possible to see on *Fig. 5.* In the presence of GO tested material we can see decrease of optical density during incubation period; the same pattern also shows rGO tested material [22].

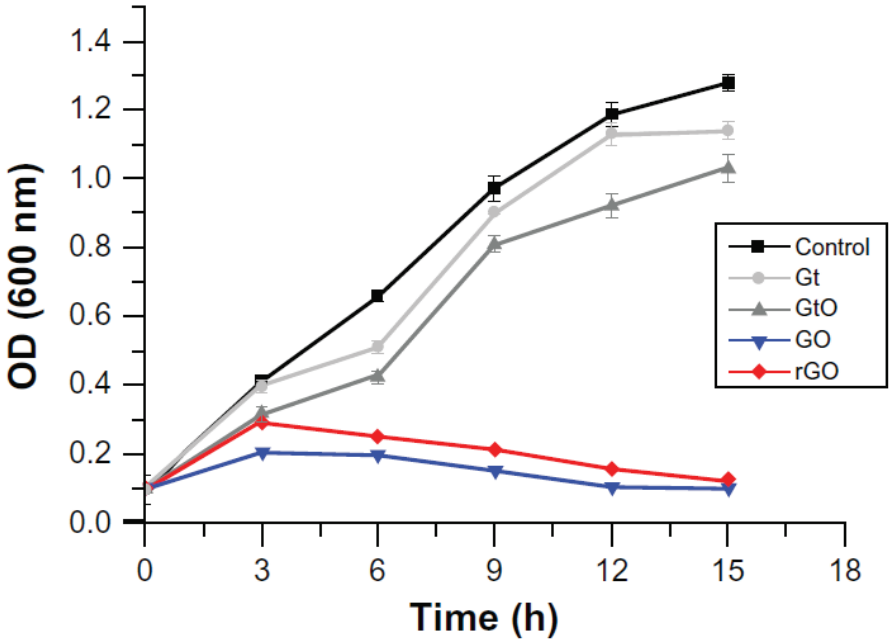


*Fig. 5 Effect of various graphene materials (Gt, GtO, GO, and rGO) on growth of Pseudomonas aeruginosa; control sample without tested material [22].*

On the other hand Ruiz *and al.* published their results which show that in presence of GO bacterial cultures grows faster and to a higher optical density than without GO. This report as first one describes no antimicrobial activity or cytotoxic properties of GO materials. Instead argues graphene oxide is a general growth enhancer that acts as a scaffold for cell surface attachment, proliferation and biofilm formation [8]; more in section 2.6. Biofilms.

Further we will deal with physiochemical properties of carbon-based materials and their potential influence on antimicrobial activity of these materials. The published fact is that physiochemical properties affect the antimicrobial activities of nanomaterials [47]. These important and widely studied properties for example are: electronic properties, size, impurities, concentration, solution chemistry, incubation time and functionalization [15].

The size dependence is one of the leading. Lyon *et al*. compared the antibacterial activities of four stable fullerene water suspensions with various aggregates sizes. They found that smaller aggregates (e.g., GO) had stronger antibacterial activity than those with larger size (e.g., GtO) [15, 48, 49]. Two different types of CNTs (SWCNTs and MWCNTs) were compared also. SWCNTs have stronger antimicrobial activities than MWCNTs with larger diameter [15, 48].

Further publication ways how nanoparticles cause cell damage is that after cell deposition on graphene nanosheets, the sharp edge of graphene nanosheets may cause significant membrane stress [50]. Liu *et al*. in their thesis publish that nanosheets serve as “cutters” to disrupt and damage cell membranes, leading to the release of intracellular contents, and eventually cell death. Further discloses that a notable difference among different graphene materials is that small GO nanosheets can wrap bacterial cells, while large rGO aggregates would trap cells [7, 51].

Time-dependent and concentration-dependent antibacterial activities were also investigated.

Several studies publish that a higher concentration of carbon nanomaterials usually results in higher death rate of bacteria [15]. Liu *et al*. show in their work that the loss of *E. coli* viability progressively goes up with the increases of GO or rGO concentration [7]; also Arias *et al*. reported the antimicrobial activity of two type of SWCNT, both increase with the raise of concentration [52].

A number of studies have found, that antimicrobial activity of CNTs is also time dependent and that longer incubation time increases the antimicrobial effect of CNTs [15]. It was indicated the loss of *E. coli* viability steadily increases with extending incubation time. Comparing GO and rGO dispersions, GO dispersions have much higher antibacterial activities than rGO dispersions at all tested incubation intervals, as we can see of *Fig.6* [7].

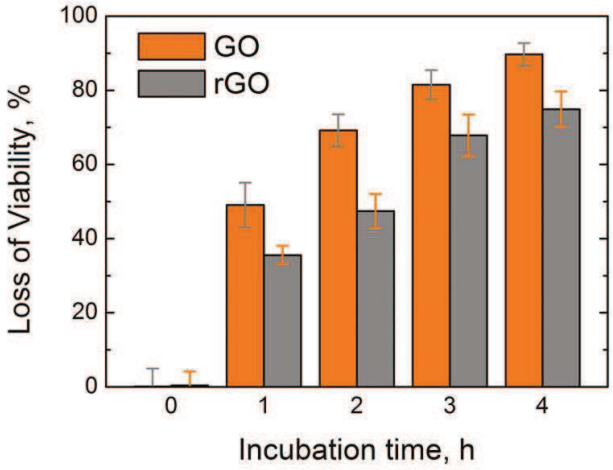


*Fig. 6 Time-dependent antibacterial activities of GO and rGO dispersions (80 μg/mL) expressed in Loss of viability (%) of E. coli cells [7].*

In contrast Kang *et al*. incubated *Bacillus subtilis* cells with CNTs and found the inactivation rate of *Bacillus subtilis* increases with extend of incubation time from 1 hour to 4 hours [53].

Antibacterial activity of carbon nanomaterials is also connected with metal residues presence. These residues result from catalysts used for CNT synthesis and can have strong toxicity on mammalian cells [54].

Next, it has been mentioned that carbon nanomaterials can be functionalized with different surface groups, which may change the antimicrobial activity of these nanomaterials [15].

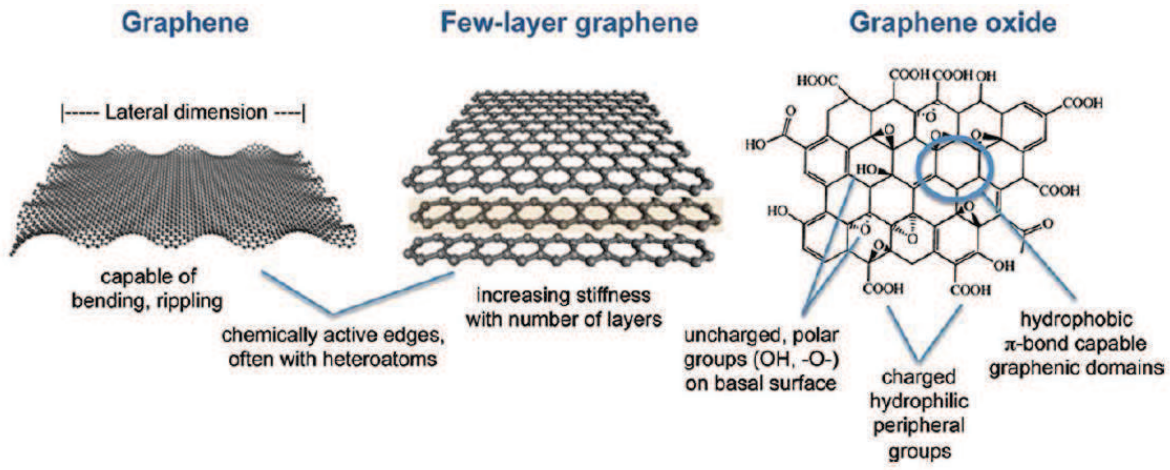


*Fig. 7 Three basic types of carbon-based materials [55]*

Lastly, there is possible to find the publication which notes that under similar concentration of nanomaterials and under similar incubation conditions, GO dispersion shows the highest antibacterial activity, sequentially followed by rGO, Gt, and GtO. Scanning electron microscope (SEM) and dynamic light scattering analyses show that GO aggregates have the smallest average size among the four types of materials and moreover SEM images display that the direct contacts with graphene nanosheets disrupt cell membrane [7].

### 2.4.1 Antimicrobial mechanism of CNMs

It is important to clarify the antimicrobial mechanism of CNMs. Then it will be possible to include these materials in a wide range of applications without causing problems to human beings and environment.

There is a three-step antimicrobial mechanism, previously predicated for carbon nanotubes, which is applicable also to graphene-based materials. In this mechanism, there are initial cell deposition on graphene-based materials, membrane stress caused by direct contact with sharp nanosheets, and the ensuing superoxide anion-independent oxidation included. It is assumed that physicochemical properties of graphene-based materials (such are density of functional groups, size, and conductivity) can be precisely tailored to either reducing their health and environmental risks or raising their application potentials [7].

Wang *et al*. reported the description of possible mechanism of GO's cytotoxicity includes first of all: attachment of GO to the surface of human cells, providing a stimuli signal to the cells. The signal is transduced inside the cells and the nucleus, leading to down-regulation of adhesion-associated genes and corresponding adhesive proteins, subsequent in decline in cell adhesion and causing cells to detach, float, and shrink in size; at the same moment, GO enters into cytoplasm by endocytosis pathway, chiefly situated in the lysosomes, mitochondrion, endoplasm and cell nucleus, may disturb the course of cell energy metabolism and gene transcription and translation, and lastly result in cell apoptosis or death [2].

It has also been reported that bacteria in contact with SWCNT networks have much less tendency to biofilm formation. It is possible to suggest, that numerous contacts between CNTs

and bacteria are necessary for the bacterial death. Chemical effects of CNTs on bacteria may be more important than physical damages and oxidative stress caused by presence of CNTs could be a major antimicrobial agent [48, 56].

Liu *et al*. concluded that the bacterial cytotoxicity may be attributed to both membrane and oxidative stress and the three step antibacterial mechanism is applicable to graphene-based materials. In general, graphene materials, containing a higher density of functional groups, and have smaller size, have more chances to interact with bacterial cells, leading in cell deposition. Graphene nanosheets can induce membrane stress by disrupting and damaging cell membranes, leading to cell death when they come into direct contact with the cell [7].

## 2.5 APPLICATIONS OF CARBON-BASED NANOMATERIALS

Nanomaterials due to their unique properties have been already applied in many fields of human life. One of the most attractive properties of nanomaterials is their antimicrobial activity [6]. Antimicrobial nanoparticles applications are possible to see on *Fig 8.*

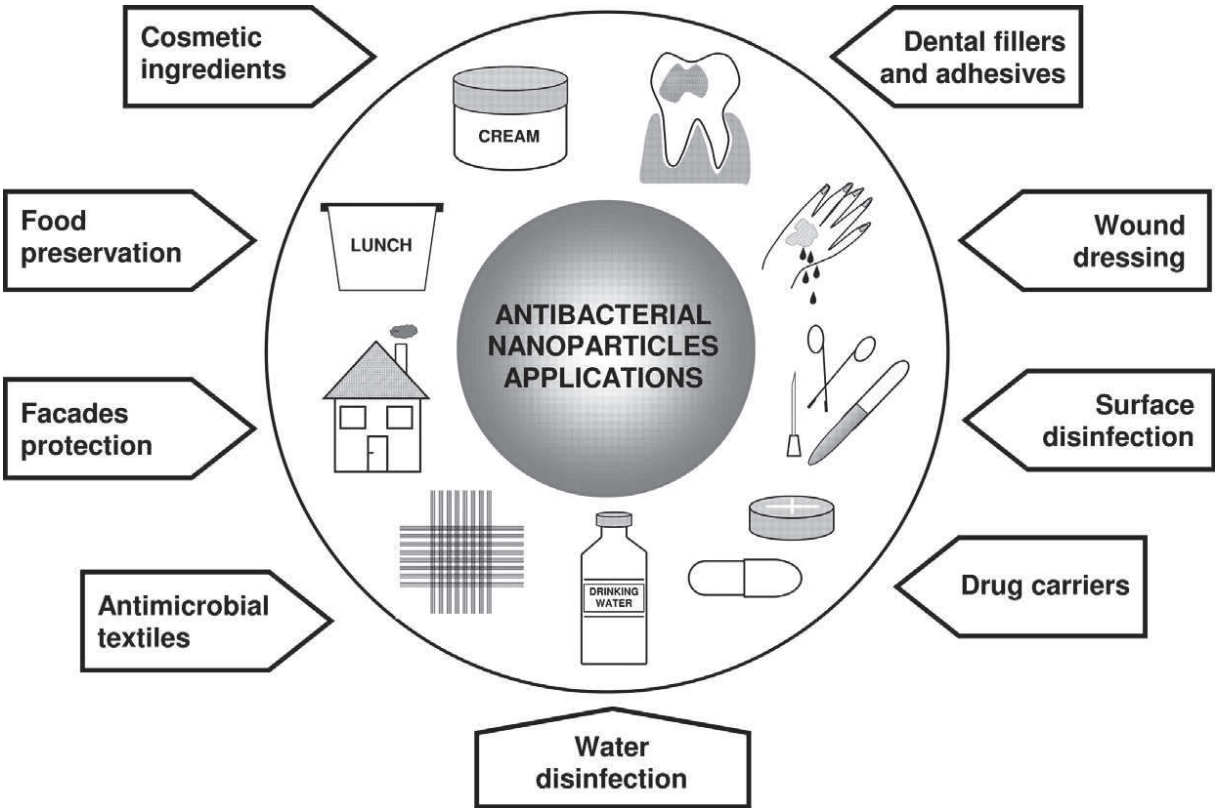


*Fig. 8 Schema of possible application of antibacterial nanoparticles [6]*

Very important application of carbon-based fillers is using as fillers into the composites, which improves the properties of these materials [5].

Graphene is one of the most important and widely studied representatives of nanomaterials. Furthermore, there are much more possible applications of graphene-based materials in other industrial branches. Graphene and graphene oxide are widely studied for

many applications such as energy storage, sensors, nanoelectronics, nanocomposits and plenty applications in biomedical area [1].

### 2.5.1 Biomedical applications of graphene-based nanomaterials

There are some anxieties about the potential toxicity and biocompatibility from the scientific community and the general public, which should be precisely investigated before in vivo studies and potential clinical translation. On the other side there is a great enthusiasm about biomedical applications of graphene-based nanomaterials [1].

The assessment of any possible toxicological side effects should be processed with the development of graphene-based nanomaterials for biomedical applications [1].

The crucial question is how to modify and functionalize graphene and its derivatives so that they do not exhibit any toxicity. Next, how can be graphene possibly cleared from the body over time, and the best use of graphene for biomedical applications [1].

The limited early studies on GFNs pointed to their potential usage as biosensors [58], tissue scaffolds [59, 60], carriers for drug delivery [61] or gene therapy [62], antibacterial agents [63], and bioimaging probes [3, 61, 64, 65]. In the field of biomedical applications, the major benefit and advantage of GFNs is their high specific surface area in comparison with other nanomaterials. This area allows high-density biofunctionalization or drug loading. Due to 2D structure of graphene material, both sides of a single graphene sheet is possible to use as a substrate for the controlled addition or adsorption of molecules and functional groups. For improving biocompatibility and colloidal stability and to impart specific biological activity to GFNs, both covalent and non-covalent surface modification has been used [3].

The main aim of tissue engineering is to replace diseased or damaged tissue with biologic substitutes that can restore and maintain normal function [66]. The mechanical abilities of graphene such as high elasticity, flexibility, and adaptability to flat or irregular surfaces [67–69] are suitable for the structural reinforcement of biocompatible films, hydrogels, and other scaffold materials, which are frequently used for tissue engineering. Hydrogel composites are very similar to soft tissue. Due to this similarity, these composites have been widely studied as scaffolds or cell-encapsulating fillers to generate or repair tissues such as skin, bladder, cartilage, and bone [70].

The next potential for use is molecular imaging with graphene-based nanomaterials [1]. The branch of molecular imaging, ‘‘the visualization, characterization and measurement of biological processes at the molecular and cellular levels in humans and other living systems’’ [71], has expanded enormously over the last ten years. Molecular imaging takes advantages of traditional imaging techniques and also introduces molecular imaging agents to measure the expression of indicative markers at different phases of disease [72].

The internal chemical and physical properties, such as ultrahigh surface area and large sp2 hybridized carbon area, promise that graphene-based nanomaterials could be carriers for efficient drug and gene delivery [1]. It was proved that GO can be used for loading (via π–π stacking) and delivery of aromatic water-insoluble cancer drugs [73]. A wide variety of nanomaterials have been examined for gene delivery and gene therapy usages. One main challenge of gene therapy is the development of a safe gene vector which could be able to

protect DNA from degradation and enable cellular uptake of DNA with great efficiency. It has been shown, that graphene could to bind to single-stranded DNA effectively but not double-stranded DNA [1].

Additionally, graphene is also able to protect oligonucleotides from cleavage by enzymes [1].

And finally the most feasible usages of graphene-based materials in nanomedicine will be in branch of cardiovascular diseases. Here is a lower biological barrier for the efficient delivery of nanomaterials. The feasible usages could be oncology also, where the leaky tumor vasculature can allow for better tissue penetration than in normal organs/ tissues [1].

As in other areas of science, it is necessary to know the risks of the use of applied materials to human health.

## 2.6 BIOFILMS

Ruiz *and al.* published their results which show that in presence of GO material bacterial cultures grow faster and to a higher optical density than without GO. Furthermore bacterial cultures produce dense biofilm in the presence of GO. By scanning electron microscopy (SEM) analysis was shown that the analyzed precipitate was built by a thick bacterial biofilm containing a large mass of aggregated cells and extracellular polymeric material. The massive amount of cells observed in the biofilm indicates that there is a direct effect of GO in bacteria proliferation when colloidal GO is added to liquid media [8].

Generally microbes build complex multicellular communities (biofilms) through increase production of extracellular polymeric substances [74]. Biofilm is possible to characterize by elevated content of EPS and much as 50% of total organic matter in biofilm consists of EPS [75]. Biofilms are thickly packed multicellular societies of microbes attached to a surface or interface. Bacteria seem to introduce biofilm formation in response to specific environmental feedbacks such as nutrient and oxygen accessibility [76]. Biofilms offer their member cells several welfares, among which protection from environmental invectives and attacks is foremost [77].

The biofilm has a variety of disadvantages, mainly in industry or pharmacy. They are the source of persistent infections of many pathogenic microbes, next are responsible for dental caries and nosocominal infections, as well as a plenty of other infections and diseases [78]. Industrially, biofilms are also detrimental in many cases. For instance, natural biofilms can reduce heat transfer in heat exchangers and cooling towers [79], decompose reverse osmosis membranes [80], corrode metal surfaces, and contaminate food processing equipment [81]. With the cells embedded in a polysaccharide matrix, biofilms are highly resistant to antibiotics and have higher genetic transformation frequencies than planktonic cells [82].

These matrixes are also an ideal place for exchanging genetic material and maintaining a large and accessible gene pool. Horizontal gene transfer is facilitated, since the cells are maintained in close proximity to each other, are not fully immobilized, and can exchange genetic information [83].

Biofilms share an important structural feature: their constituent cells are bound together by an extracellular matrix that mainly consists of macromolecules, including polysaccharides,

proteins, and nucleic acids, that are produced by the cells themselves [84]. Extracellular matrices have been shown to play essential roles in the establishment and maintenance of biofilm structure [74].

A wild strain of the Gram positive bacterium *Bacillus subtilis* is capable of forming such a matrix [74, 85]. These wild strains form heavy biofilms both at liquid/air interfaces and on solid surfaces [86]. In standing liquid medium, *Bacillus subtilis* cells switch from a submerged, highly motile planktonic state in which the bacteria swim as single cells, to a non-motile state in which the cells grow as bundled chains that rise to the surface and form a robust pellicle. On the surface of agar plates, the cells form colonies with elaborate architecture, including aerial structures that resemble fruiting bodies and that preferentially produce spores at their tips [86]

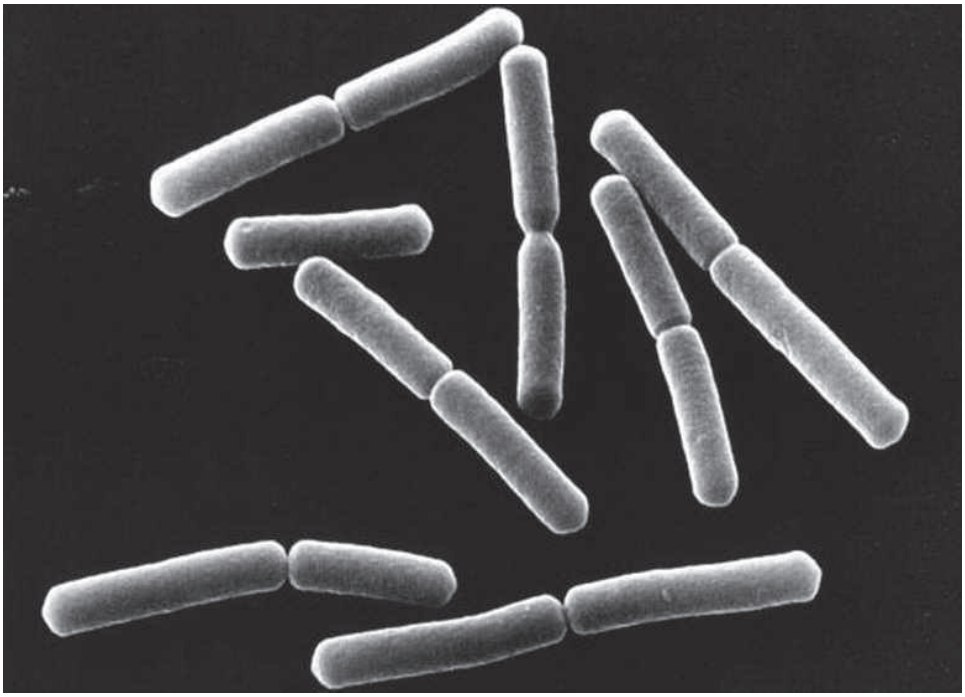

*Fig. 9 Bacillus subtilis [87]*

Some wild strains of *B. subtilis* produce elaborate biofilms in which spore formation takes place preferentially at the tips of aerial structures that protrude from the surface of the community [86]. There are a plenty of genes that influence biofilm formation in *B. subtilis* [86, 88–90]. Biofilm formation and sporulation are also connected in that both procedures are dependent on *Spo0A*, the dominant regulator for entry into sporulation [86, 88, 91]. One of the major components of the biofilm, the exopolysaccharide is produced by enzymes encoded by the *epsA-O* operon and the gene encoding TasA is situated in the *yqxM-sipW-tasA* operon. Both operons are under the control of the repressor SinR [92]. There are more very important genes, which involved on biofilm production, genes that are putatively involved in EPS production (*yhxB* and the 15-gene-long *yveK-yvfF* operon), which is herein renamed *epsA-0*, a gene encoding a putative phosphatase (*yqeK*), a gene involved in the production of the surfactant, surfactin ( s*fp* ), a gene encoding a signal peptidase (*sipW*), a gene encoding an

ABC transporter subunit (*ecsB*), and two genes of largely unknown function whose inferred products exhibit substantial amino acid sequence similarity to each other (*ylbF* and *ymcA*) [85].

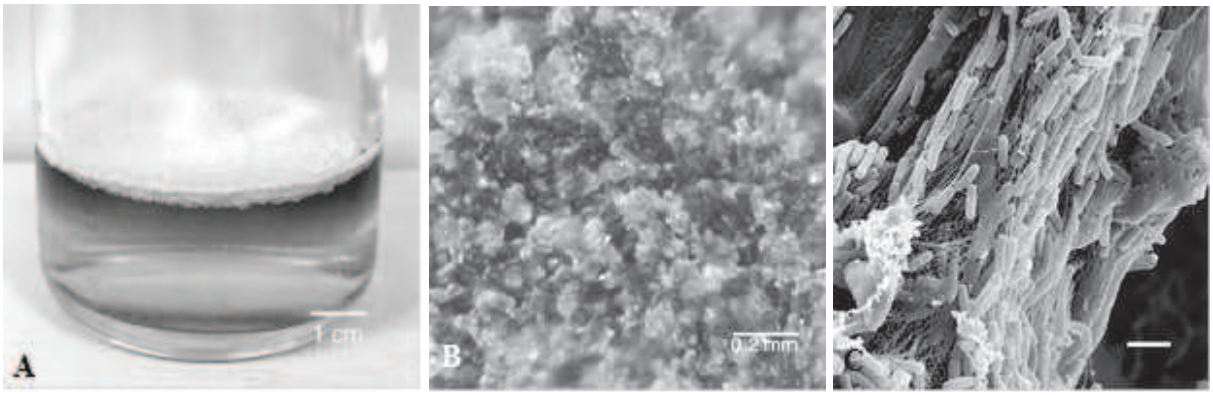


*Fig. 10 Biofilm formed by wild type of B. subtilis. Side view (A); optical microscopic observation (B); SEM observation (C) [76]*

The organizing principle afforded by surfaces appears to have been commonly exploited by microbes during the course of evolution. Most microorganisms seem to be capable of biofilms formation of some sort or another. Molecular genetic approaches have begun to explain the mechanisms by which microbes build such communities. Based on comparison of biofilm formation by many different organisms was found that the extracellular matrix is absolutely essential for biofilm structure [74, 84]. Like several other microbes, *Bacillus subtilis* builds a matrix, containing both exopolysaccharides and protein [74].

It is necessary to said that mutations that eliminate EPS production have a serious impact on biofilm formation, while those that eliminate TasA tend to have less serious impact. Most importantly is finding that elimination of both EPS and TasA leads to a particularly severe phenotype in which pellicle formation is entirely prevented. These results are reported as an indication that EPS and TasA are the two most important matrix components in *Bacillus subtilis* biofilms [74].

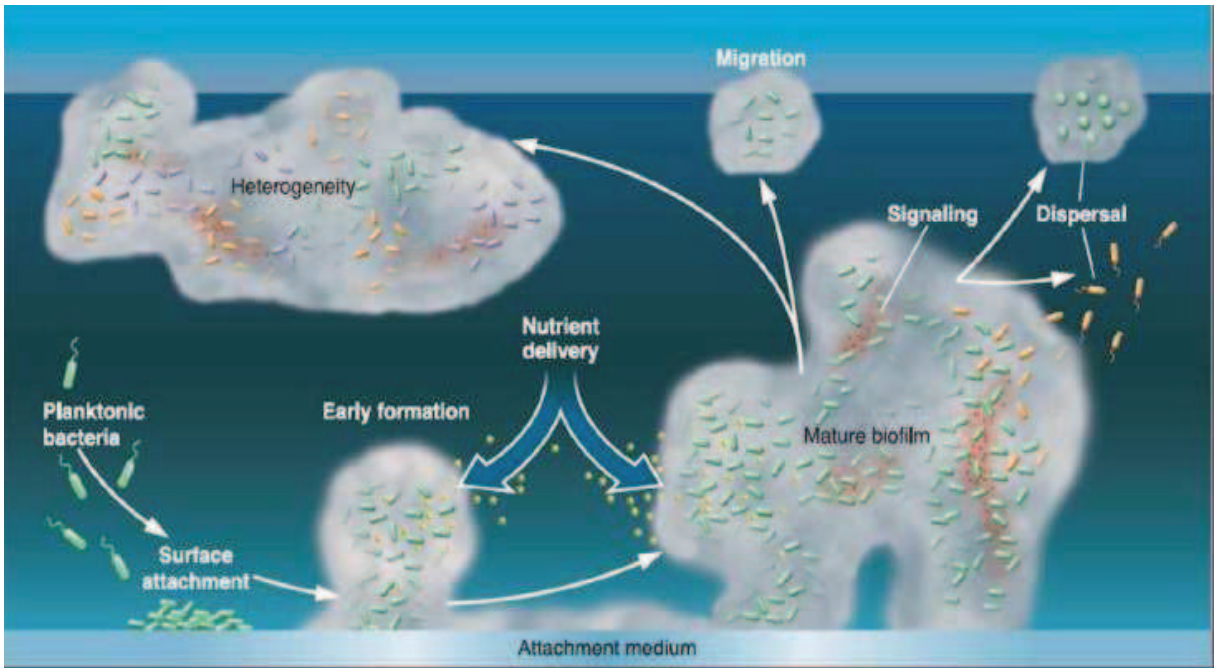


*Fig. 11 View of the dynamic nature of a biofilm community [93]*

## 2.7 EPS (EXTRACELLULAR POLYMERIC SUBSTANCES)

With biofilm formation related presence of EPS in the environment, because the EPS are an important part of the biofilm or may be produced in stressful situation for cell that can be immediate contact with nanomaterial.

EPS produced by bacteria are molecules released in reply to the physiological stress encountered in the natural environment. EPS are structural constituents of the extracellular matrix in which cells are fixed during biofilm development and the chemical nature and functions of these EPS are dependent on the genetic expression of the cells within each biofilm. Although some bacterial matrices have been characterized, understanding of the function of the EPS is relatively limited, particularly within the *Bacillus* strain genus [94].

The main function of the EPS is the regulation of the immediate conditions of life of biofilm cells living in this microenvironment by affecting several features, such are: porosity, density, water content, charge, sorption properties, hydrophobicity, and mechanical stability [83, 95]. EPS is possible to describe as biopolymers of microbial origin in which biofilm microorganisms are fixed. Despite to common belief, EPS are definitely more than only polysaccharides; EPS include, in addition, a wide variety of proteins, glycoproteins, and glycolipids, and in some cases, unexpected amounts of extracellular DNA (e-DNA) and in environmental biofilms, polysaccharides are often only a minor component [83, 96]. All EPS biopolymers contain high level of water and form a matrix, which keeps the biofilm cells together and retains water. This matrix interacts with the environment, e.g. by attaching biofilms to surfaces and through its sorption properties, which allow for sequestering of

dissolved and particulate substances from the environment, providing nutrients for biofilm organisms [83, 97].

It is important to mention the *psl* operon, which is required in order to maintain the biofilm structure after attachment. In environmental biofilms, it is enormously difficult to separate and characterize specific polysaccharides in detail [98].

The EPS proteins do not play the structural role only; they can act as enzymes also [99].

There is possible to different two types of EPS. There are EPS which are bound tightly (TB-EPS) with solid surfaces and which are soluble (LB-EPS), also called slime polymers, LB-EPS is possible to move freely between sludge flocs and surrounding liquor [100, 101]. Bacteria in the suspension and floc matrix are likely to have a dynamic double-layered EPS structure of loosely bound EPS (LB-EPS) diffused from the TB-EPS that surrounds the cells [102]. The LB-EPS may work as the primary surface for cell attachment and flocculation. But, most previous experimental work on EPS has not specifically explain the role of LB-EPS in cell adhesion; and in fact, methods used for EPS extraction usually consist of thorough washing followed by harsh extraction, and the EPS that was determined in many previous studies was actually the total EPS or TB-EPS. Thus, only a little information is available which distinguishes the proportions of the two types of EPS and their possibly different effects on the surface behavior of biomass [103, 104].

There was reported method for separation these two types of EPS: the heating extraction method was modified to include a mild extraction step for extracting the LB-EPS and a harsh extraction step for extracting the TB-EPS from a sludge suspension [103].

In addition to EPS, by cells are also secreted the soluble cellular components (SMP) during synthesis of biomass or are excreted for uncertain reasons. The SMP mainly contains polysaccharide, some lipids, and a certain amount of humic substances, while the LB-EPS contains mainly polysaccharide, proteins, lipids, and some humic substances. The TB-EPS is mainly composed of polysaccharide and some lipids. The measured amounts of humic substances in LB-EPS and TB-EPS samples were less in relative fraction than in SMP samples [101].

When focusing on specific microorganism, we should mention bacterium *Bacillus subtilis* again. Because some widely strains of *Bacillus subtilis* secrete a wide variety of EPS [105]. Important is to publicize that greater amounts of free EPS are produced during the stationary phase and that in EPS produced by BS we can find structural, sorptive, surface-active and active EPS [105]. During vegetative growth the secretion of EPS plays an important role in community survival [105].

Finally, it should be noted that the EPS matrix could be considerably more than simply the glue for biofilms. Rather, it appears EPS a highly sophisticated system, which endows the biofilm mode of life with particular, effective features [83].

# 3 EXPERIMENTAL PART

## 3.1 MATERIALS AND EQUIPMENT

### 3.1.1 Instrumentation

- Analytical Balance; AND GR-202-EC, Japan
- Autoclave; Vaspoteri – Brněnská medicínská technika, Czech Republic
- Automatic pipettes; Hirschmann, Biohit Proline
- Centrifuge; Eppendorf Concentrator 5301, Hamburg, Germany
- Desiccator; Simax, Czech Republic
- Flow-oven; Memmert, Germany
- Fridge; Samsung, South Korea
- Incubator; Heidolph – Germany
- Kitchen stove, ETA
- Laboratory Balance; Scaltec, USA
- Laminar box AURA mini; Biotech, Czech Republic
- Mikrocentrifuge – Eppendorf centrifuge 5417R, Germany
- Polypropylene peak ZipTip ®, Millipore, C18
- Spectrophotometer; UV/VIS HELIOS DELTA - Thermospectronic, UK
- Thermostat; Huber, Germany
- Vortex; Heidolph, REAX top, Germany
- Spektrofotometer SPEKOL 1300, Analytik Jena AG, Germany
- Incubator 1000, Heidolph, Germany

### 3.1.2 The reagents

- Ammonium molybdate tetrahydrate $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$ - Lachema, Czech Republic
- Bovine serum albumin (BSA) - Sigma Aldrich Germany
- Copper (II) sulphate pentahydrate $CuSO_4 \cdot 5\ H_2O$ - LachNer, Czech Republic
- D-glucose $C_6H_{12}O_6$ - Lachema, Czech Republic
- Distilled water $H_2O$ - BUT Faculty of Chemistry, Czech Republic
- Ethanol $C_2H_5OH$ - Merci, Czech Republic
- Folin-Ciocault Reagent - Czech Republic
- Magnesium sulphate $MgSO_4$ - LachNer, Czech Republic
- Peptone - Himedia Laboratories Limited
- Phenol $C_6H_6O$ - LachNer, Czech Republic
- Potassium dihydrogen phosphate $KH_2PO_4$ - Lach Ner, Czech Republic
- Sodium azide p.a., LachNer, Neratovice
- Sodium bicarbonate $NaHCO_3$ - Lachema, Czech Republic
- Sodium carbonate $Na_2CO_3$ - Lachema, Czech Republic
- Sodium chloride NaCl - LachNer, Czech Republic

- Sodium hydroxide NaOH - LachNer, Czech Republic
- Sodium potassium tartrate tetrahydrate $C_4H_4O_6KNa \cdot 4 H_2O$-LachNer, Czech Republic
- Sodium sulphate anhydrous $Na_2SO_4$ - LachNer, Czech Republic
- Sulfuric Acid $H_2SO_4$ 96%
- Tween 80 - Polyoxyethylen-Sorbitan-Monooleat - Switzerland
- Yeast extract - HiMedia Laboratories Limited, India

### 3.1.3 Biological material

- *Bacillus subtilis* CCM 1999, culture was obtained from the Czech Collection of Microorganisms (CCM), Masaryk University Brno, Faculty of Science, Czech Republic.
- *Yarrowia lipolytica* CCY 29-26-52, culture was obtained from the Slovak Collection of Yeast, SAV, Slovakia.

### 3.1.4 Nano-material

Carbon nanomaterials used in this work were obtained from Department of Inorganic Chemistry, Institute of Chemical Technology, Prague.

There were used three different carbon nanomaterials with abbreviation "A", "B" and "C", with a variation in the spacing between the graphene layers and with different degree of oxidation.

Material "C", which is prepared by thermal reduction, has the largest particles, the largest distance between graphene layers respectively and also exhibits a lower degree of oxidation.

### 3.1.5 Culture media

- NBG (Nutrient Broth + Glucose):

| | |
|---|---|
| Peptone | 30 g/L |
| Yeast extract | 10 g/L |
| Sodium chloride | 5 g/L |
| D-glucose | 20 g/L |

- MPA (Meet-Peptone Agar) - Himedia Laboratories Limited
- BM+TW 20 - basal medium with Tween 20:

| | |
|---|---|
| Peptone | 5 g/L |
| $MgSO_4$ | 0.1 g/L |
| $K_2HPO_4$ | 1 g/L |

### 3.1.6 Solutions and their preparations

#### *3.1.6.1 Solutions for Lowry protein assay*

- Solution of bovine serum albumin (1 mg/mL).
- Reagent A consists of 2% $Na_2CO_3$ (20 g/1000 mL), 0.05%, $KNaC_4H_4O_6 \cdot 4H_2O$ (0.05 g/1000 mL), 0.1 M NaOH (4 g/1000 mL).

- Reagent B consists of 0.1% $CuSO_4 \cdot 5H_2O$ (1 g/1000 mL).
- Reagent C consists of 45 mL of solution A + 5 mL solution B (newly diluted in proportion 9:1).
- Reagent D consists of 1 vol Folin-Ciocalteu reagent diluted with 1.6 vols water.

#### *3.1.6.2 Solutions for Nelson-Somogyi Assay*

- Nelson-Somogyi I consists of $C_4H_4O_6KNa \cdot 4\ H_2O$ (60g/1000 mL), $NaHCO_3$ (80g/1000 mL), $Na_2CO_3$ (90g/1000 mL), $Na_2SO_4$ (240 g/1000 mL).
- Nelson-Somogyi II consists of $CuSO_4 \cdot 5\ H_2O$ (20g/ 1000 mL), $Na_2SO_4$ (180g/1000mL).
- Nelson-Somogyi III consists of $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$ (55g/1000 mL), 96% $H_2SO_4$, $Na_2HAsO_4 \cdot 7H_2O$ (12g/1000 mL).

## 3.2 METHODS

### 3.2.1 Preparation of the microorganism cultivated in a nutrient medium

#### *3.2.1.1 Recovery of Bacillus subtilis from gelatin discs*

During the recovery of bacterial culture, *BS* was transferred on slant agar (MPA) from gelatin discs. Firstly, discs were moved into the laboratory temperature environment for 10 minutes. Secondly, disc was transferred aseptically to sterile water in test tube using inoculation loop. Suspension poured down all the surface of slant agar due to the inclination of test tube. Subsequently the *BS* culture has been cultivated in thermostat for two days at 30 °C. *BS* culture for inoculum preparation was cultivated on MPA agar at 30 °C for three days before using.

#### *3.2.1.2 Preparation of inoculum of Bacillus subtilis*

There were used two slant agars for preparation of inoculum. 1 mL of sterile distilled water was aseptically added to the *BS* culture. Afterwards the culture of *BS* was wiped by inoculating loop on the wall below the liquid level. Thereafter slant agars were mixed by vortex and theirs liquid contents were transferred to a sterile Erlenmeyer flask containing 100 mL of NBG medium. Before using, thus prepared inoculum was shacked 24 hours on a shaker (160 rpm, laboratory temperature). The prepared inoculum ($1290 \cdot 10^6$ CFU/mL) was used for inoculation of L-tubes.

#### *3.2.1.3 Preparation of inoculum of Yarrowia lipolytica*

The *YL* culture was grown on slant wort agar for 3 days at 28 °C. One pace of this slant wort agar was used for preparation of inoculum. The culture of *YL* was suspended in 10 mL of sterile distilled water in test tube by inoculating loop. The inoculum ($540 \cdot 10^6$ CFU/mL) was ready to use without following shaking.

### 3.2.2 Determination of standard curves

#### *3.2.2.1 Determination of standard curve of bovine serum albumin*

Bovine serum albumin (BSA) solution with concentration 1 mg/mL was used. A calibration range with following volume of BSA (0.0 (blank); 0.1; 0.2; 0.3; 0.4; 0.5 mL) was prepared by diluting in water according to *Tab. 1*. Subsequently, reagent C (5 mL) was added to the reaction mixture. The reaction mixtures was stirred and incubated for 10 minutes at laboratory temperature. Then the reagent D (0.5 mL) was added to the reaction mixture. Absorbance of these solutions was measured spectrophotometrically at a wavelength of 750 nm after incubation for 30 minutes at laboratory temperature.

*Tab. 1 Standard curve of BSA*

| tube | 1 | 2 | 3 | 4 | 5 | Blank |
|---|---|---|---|---|---|---|
| Albumine [mL] | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 | 0.0 |
| Distilled water [mL] | 0.9 | 0.8 | 0.7 | 0.6 | 0.5 | 1.0 |
| Reagent C [mL] | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 |
| Reagent D [mL] | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Albumine [mg/mL] | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 | 0.0 |

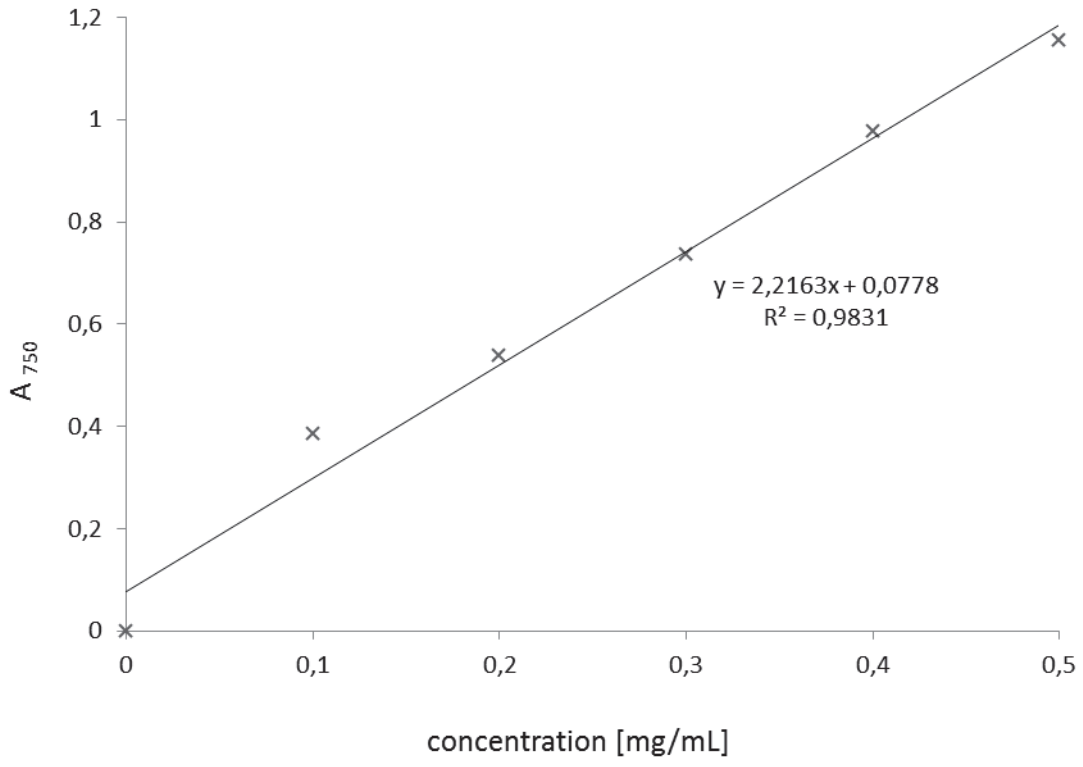


*Fig. 12 Standard curve of BSA*

#### *3.2.2.2 Determination of standard curve of glucose for determination of reducing substances by Nelson-Somogyi Assay*

Standard glucose solution with concentration 0.2 g/L was used. A calibration range with following volume of glucose (0.0 (blank); 45; 68; 90; 113; 135; 158; 200; 300; 400; 500; 600; 800; 1000 μL) was prepared by diluting in water according to *Tab. 2*. Subsequently, mixture of reagents Somogyi-Nelson I and Somogyi-Nelson II (4:1) was added (1.0 mL). The reaction mixture was boiled for 10 minutes. Finally, the test tubes were cooled to laboratory temperature and 1.0 mL of the Somogyi-Nelson III reagent and 7.0 mL of distilled water were added. Then, the absorbance at 530 nm was measured.

*Tab. 2 Standard curve of glucose for determination of reducing substances by Nelson-Somogyi Assay*

| tube | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | Blank |
|---|---|---|---|---|---|---|---|---|---|
| Glucose [μL] | 68 | 113 | 135 | 158 | 500 | 600 | 800 | 1000 | 0 |
| Distilled water [μL] | 932 | 887 | 865 | 842 | 500 | 400 | 200 | 0 | 1000 |
| S.-Nelson I and II [mL] | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| S.-Nelson III [mL] | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Distilled water [mL] | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 |
| Glucose [mg/mL] | 0.014 | 0.023 | 0.027 | 0.032 | 0.100 | 0.120 | 0.160 | 0.200 | 0.000 |

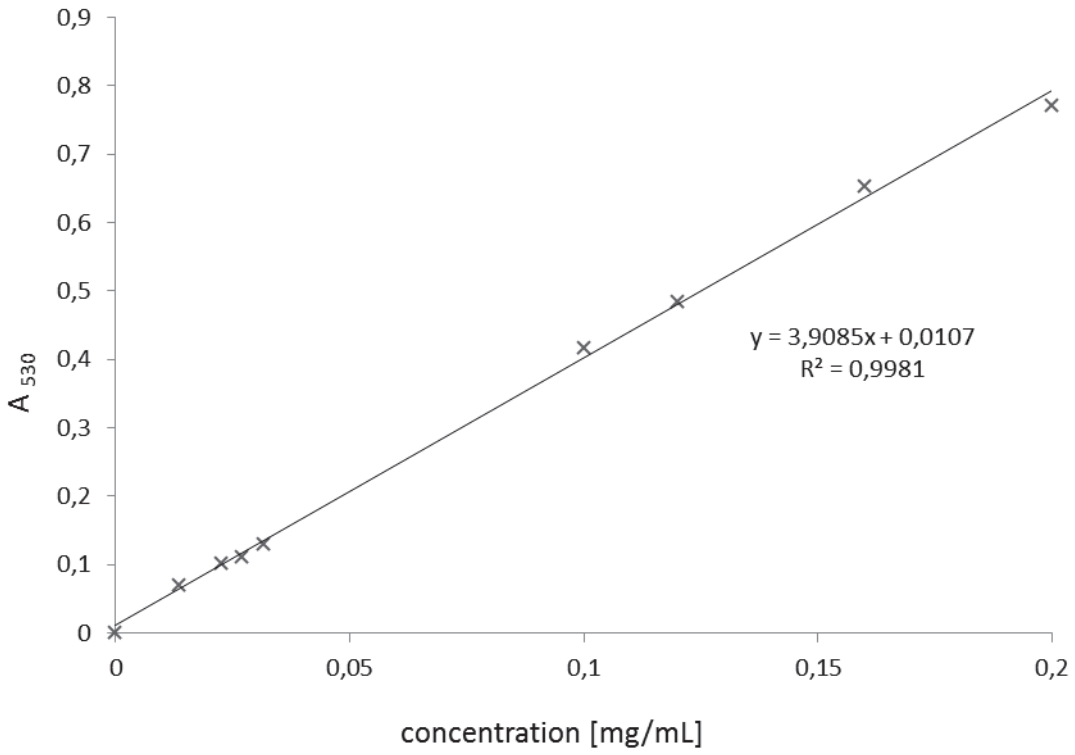


*Fig. 13 Standard curve of glucose for determination of reducing substances by Nelson-Somogyi Assay*

#### *3.2.2.3 Determination of standard curve of glucose for determination of total soluble sugars by Dubois Method*

Glucose solution with concentration 0.1 g/L was used as a standard. A calibration range with following volume of glucose (0.0 (blank); 40; 50; 62.5; 150; 250; 400; 500 µL) was prepared by diluting in water according to *Tab.3*. Subsequently, phenol (0.5 mL) and concentrated sulfuric acid (2.5 mL) were added to reaction mixture. The absorbance of these solutions was measured spectrophotometrically at a wavelength of 490 nm.

*Tab. 3 Standard curve of glucose for determination of total sugars by Dubois Method*

| tube | 1 | 2 | 3 | 4 | 5 | 6 | 7 | Blank |
|---|---|---|---|---|---|---|---|---|
| Glucose [mL] | 40 | 50 | 62,5 | 150 | 250 | 400 | 500 | 0 |
| Distilled water [mL] | 460 | 450 | 437.5 | 350 | 250 | 100 | 0 | 500 |
| Phenol [mL] | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Sulfuric acid [mL] | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 |
| Glucose [mg/mL] | 0.080 | 0.100 | 0.125 | 0.300 | 0.400 | 0.800 | 1.000 | 0.000 |

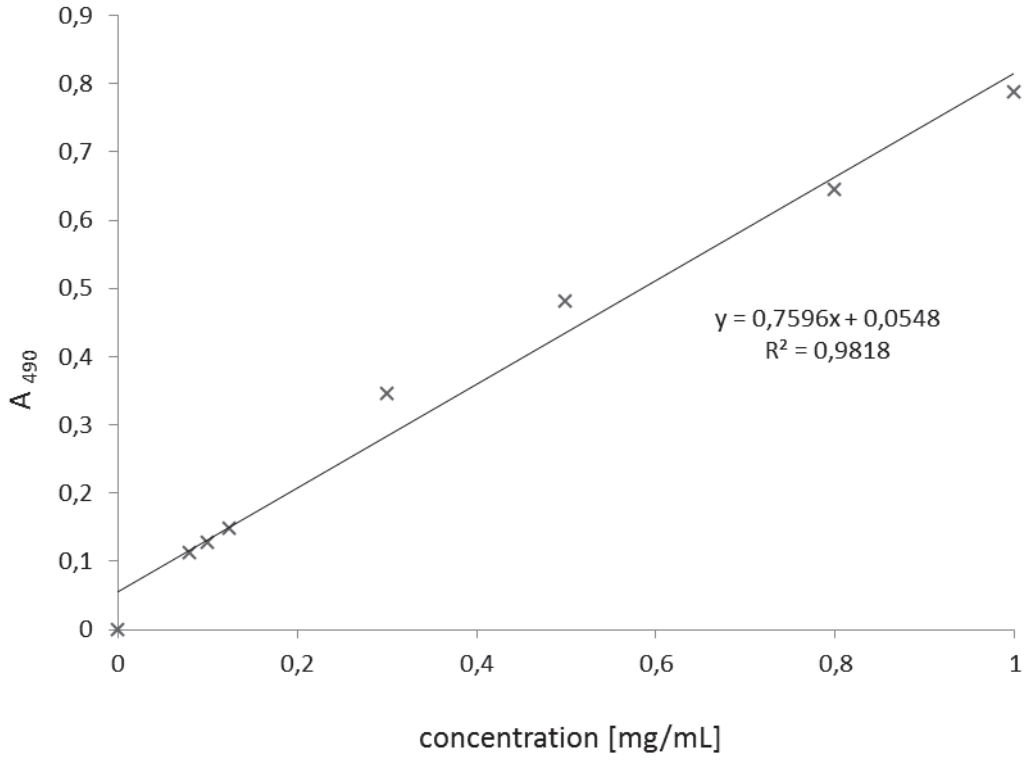


*Fig. 14 Standard curve of glucose for determination of total sugars by Dubois Method*

### 3.2.3 Test of antibacterial activity

All experiments were performed in the L-tubes. 0.8 mg tested material (graphene oxide or graphene) was resuspended in *BS*- or *YL*–inoculated cultivation medium (6 mL). Subsequently, microorganisms were dynamically cultivated (160 rpm or 200 rpm) at 30 °C (*Bacillus subtilis*) and 28 °C (*Yarrowia lipolytica*).

Samples prepared this way were used as a bacterial suspension for determination of optical density, bacterial growth expressed as colony forming units and the biomass concentration. After centrifugation the cell free supernatant was used for determination of concentration of extracellular polymeric substances and concentration of extracellular proteins.

Control test was performed in the same way but without tested material. In each following experiment the two parallel measurements were made.

### 3.2.4 Optical density assay

Optical density was measured for growth of microorganism assessment. Samples prepared according to section 3.2.3 were used for this determination. Bacterial growth was determined spectrophotometrically (Spectrophotometer; UV/VIS HELIOS DELTA - Thermospectronic, UK) at 600 nm. The tested solution was replaced with distilled water for blank measurement.

### 3.2.5 Lowry protein assay

The Lowry method is based on a biuret method. The first component is a biuret reagent; the second is Folin-Ciocalteu agent. The Biuret method is based on the chelation of copper ions by imide structures of the polypeptide chain at alkaline pH.

Proteins are firstly treated with alkaline copper sulphate in the presence of tartrate and followed by addition of the Folin-Ciocalteu reagent. The creation of the color reaction in the Lowry procedure occurs when the tetradentate copper complexes transfer electrons to Folin-Ciocalteu reagent (phosphomolybdic/ phosphotungstic acid complex; $Mo^{+6}/W^{+6}$). After that, the reduction of the Folin-Ciocalteu reagent is measured by a blue color at 750 nm [106].

The concentration of proteins has been calculated using linear regression equation obtained by evaluation of standard curve of BSA.

Formula for calculation of the concentration of proteins:

$$c = \frac{A_{750} + c_b}{c_a}$$

| | |
|---|---|
| $c$ | concentration of enzyme [mg/mL] |
| $c_b, c_a$ | values based on linear regression equation $A = c_a \cdot c + c_b$ |
| $A_{750}$ | absorbance |

There were used bacterial suspensions prepared in section 3.2.3. for this assay. These suspensions were centrifuged (15.000 rpm, 4 °C, 5 min).

The reaction mixture consists of 250 μL of cell free supernatant and 1.25 mL of reagent C. The mixture was incubated for 10 minutes in laboratory temperature. Then, 125 μL of reagent D was added to reaction mixture and incubated for 30 minutes. The absorbance was measured at a wavelength of 750 nm. 250 μL of distilled water was used for blank.

### 3.2.6 Nelson-Somogyi Method

The Nelson-Somogyi Method is widely used for the quantitative determination of reducing sugars. The principle of this method is based on heating reducing sugars with alkaline copper tartrate and on reduction of the copper from the cupric state to the cuprous state and cuprous oxide is formed. This oxide is treated with arsenomolybdic acid and the reduction of molybdic acid to molybdenum is expressed by the blue color [106]. This color is measured spectrophotometrically at 530 nm.

The concentration of reducing substances has been calculated using linear regression equation obtained by evaluation of standard curve of glucose.

Equation for calculation of the concentration of reducing substances is a follows:

$$c = \frac{A_{530} + c_b}{c_a}$$

| | |
|---|---|
| $c$ | concentration of glucose [mg/mL] |
| $c_b, c_a$ | values based on linear regression equation $A = c_a \cdot c + c_b$ |
| $A_{530}$ | absorbance |

The suspension prepared in point 3.2.10. was added to the 0.5 mL mixture of solution Nelson-Somogyi I and solution Nelson-Somogyi II (the mixture was prepared using the ratio 4:1) in amount of 0.5 mL. This solution was mixed and heated at 100 °C for 10 minutes.

Finally the tubes were cooled to laboratory temperature and 0.5 mL of the Nelson-Somogyi III reagent and 3.5 mL of distilled water were added. Then, the absorbance at 530 nm was measured. Instead of the tested solutions, the quantity of 0.5 mL of distilled water was used for blank.

### 3.2.7 Determination of total sugars by Dubois Method

This method is based on dehydration of sugars with concentrated sulfuric acid and subsequent condensation of the resulting furfural or 5-hydroxymethylfurfural with phenol to form a colored condensation products which can be spectrophotometrically determined [106].

Suspension prepared same way as in previous method was used for this experiment. This suspension was diluted with water using the ratio 1:9 and 0.5 mL of this solution was used. Subsequently, phenol (5 %, 0.5 mL) and concentrated sulfuric acid (2.5 mL) were added to this reaction mixture. Absorbance of these solutions was measured spectrophotometrically at a wavelength of 490 nm. The tested solution was replaced with distilled water for blank measurement.

### 3.2.8 Determination of concentration of biomass

The reaction mixture consists of 50 μL of the bacterial suspension prepared in point 3.2.3 and 4.95 mL of distilled water. The concentration of biomass was measured spectrophotometrically using default program which counts amount of protein per volume unit on Spectrophotometer SPEKOL (Germany, UV-VIS method). The tested solution was replaced with distilled water for blank measurement.

### 3.2.9 Determination of concentration of extracellular proteins

In cell-free supernatant obtained by centrifugation (15.000 rpm, 4 °C, 5 min) of bacterial suspension prepared in the same way as in previous point the concentration of extracellular proteins was tested. Samples (50 μL) were diluted in distilled water to a final volume of 5 mL. The concentration of extracellular proteins was measured spectrophotometrically using default program which counts amount of protein per volume unit on Spectrophotometer SPEKOL (Germany, UV-VIS method). The tested solution was replaced with distilled water for blank measurement.

### 3.2.10 Determination of growth kinetics of *Bacillus subtilis*

Bacterial suspension with and without tested materials prepared same way as in point 3.2.3 was used for this determination, nevertheless, cultivation was carried out under 160 rpm shaking speed only. Bacterial growth was evaluated by colony counting method. Briefly, series of 10-fold cell dilutions (1 mL each) were spread onto NBG plates, and left to grow overnight at 30 °C in thermostat (Thermostat; Huber, Germany). Subsequently colonies were counted using Plating and CFU counting method.

### 3.2.11 Determination of growth kinetics of *Yarrowia lipolytica*

Yeast suspension prepared same way as in point 3.2.3. was used for this determination. Cultivation was carried out at 160 rpm shaking rate. Yeast growth was determined by cells

counting in Bürker counting chamber. Methylene blue was used as a dye for distinction live and dead cells. And only the live cells were counted for this determination.

### 3.2.12 Determination of EPS

The suspension of microbial culture with graphen or graphen oxide prepared according to section 3.2.3. was centrifuged (7.800 rpm, 4 °C, 10 min). The obtained cell free supernatant was precipitated by 18 mL of ethanol (96 %) for 24 hours. Afterwards, this mixture was centrifuged again (7.800 rpm, 4 °C, 10 min) and the precipitate was re-suspended in 10 mL of distilled water. Subsequently, concentration of proteins (by using Lowry protein assay), concentration of reducing substances (by using Nelson-Somogyi Method) and concentration of total sugars (by using Dubois Method) was determined.

# 4 RESULTS AND DISCUSSION

This study is focused on antimicrobial activity screening of carbon-based materials. Carbon-based materials could be potentially widely applied as energy storage, sensors, nanoelectronics, nanocomposites and widely applications in biomedical area, for example [1]. There is strong need of careful evaluation of these materials due to these possible applications; which means to determine microorganism growth and extracellular proteins production, mainly.

For this study three types of carbon nanomaterials referred to as “A”, “B” and “C” with a variation in the spacing between the graphene layers and with different degree of oxidation were used.

Material “C” has the largest particles, the largest distance between graphene layers respectively and also exhibits a lower degree of oxidation.

## 4.1 SCREENING OF CULTIVATION CONDITIONS

It was published, that different concentrations of tested nanomaterials have an influence on microorganisms viability and also the shaking rate could change aeration conditions and consequently viability of cells [7, 52, 107]. For screening of the most suitable cultivation conditions, two important parameters – different concentrations of tested nanomaterial, and different shaking rates were studied.

### 4.1.1 Screening of different concentrations of carbon nanomaterials

Antimicrobial effect of carbon nanomaterials could be concentration dependent. This impact was examined in several studies [7, 52]. From this point of view screening of different concentrations of tested material was performed.

For screening study in this thesis two different concentrations (0.068 mg/mL and 0.135 mg/mL) of tested material “A” were used. Tested material was incubated with *Bacillus subtilis* (160 rpm, 30 °C) for 120 hours. As a control for this experiment, cultivation of *Bacillus subtilis* without tested material was performed.

Bacterial growth and protein concentration determined by Lowry protein assay were monitored after 24, 48, and 120 hours of incubation period. Generally the determination of protein concentration was used as a supplementary method to the bacterial growth assay, because provide information about bacterial metabolism.

The effect of carbon nanomaterial concentrations on microbial growth was evaluated by the spectrophotometric measurement (600 nm) of optical density. The optical density was determined in inoculated medium mentioned above.

For higher concentration of tested materials (0.135 mg/mL) the bacterial growth significantly decreased after 48 hours of incubation in comparison with lower concentration (0.068 mg/mL) of tested material, where the growth is slightly higher than in control sample without tested material (*Fig. 15*). This finding correlate well with information published in several studies i.e. a higher concentration of carbon nanomaterials usually results in a higher

death rate of bacteria. Liu *et al*. show that the loss of *E. coli* viability progressively goes up with the increases of GO or rGO concentration [7]; also Arias *et al*. reported the antimicrobial activity of two type of SWCNT, both increase with the raise of concentration [52].

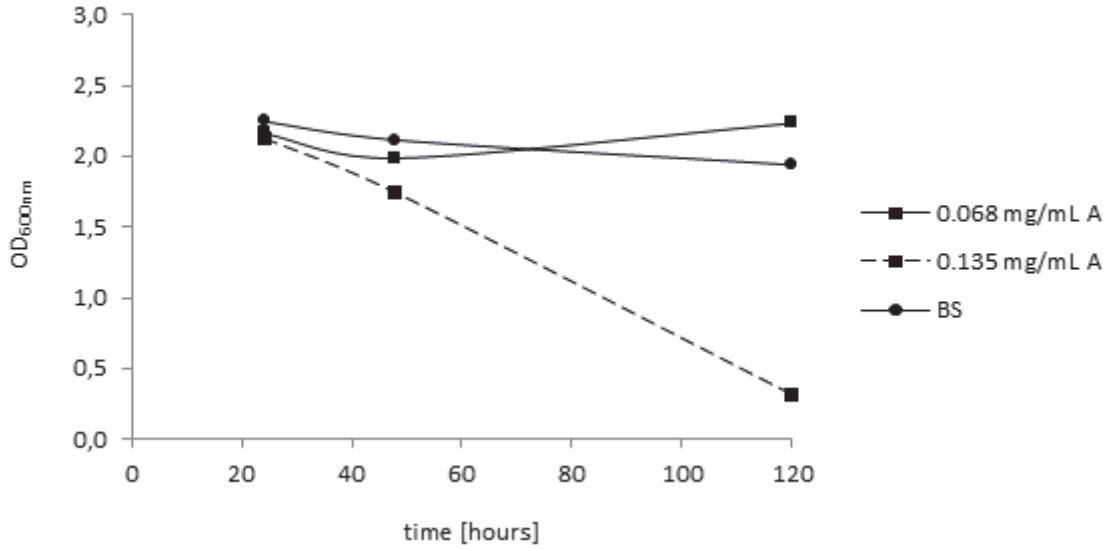


*Fig. 15 Time profile of optical density of BS in cultivation medium with and without tested carbon nanomaterials*

In the presence of both concentrations of tested material and also in control sample without carbon material, the amount of proteins during the 120 hours of cultivation did not differ so much (*Fig. 16*).

In the presence of higher concentration (0.135 mg/mL) of tested material the amount of proteins in cultivation medium is lower than in less concentrated sample probably due to reducing the efficiency of microorganism metabolism due to the influence of higher concentration of material (*Fig. 16*); this result cannot be compared with results in other studies, because the impact of higher concentration of carbon nanomaterials against microbial production of proteins has not been published yet.

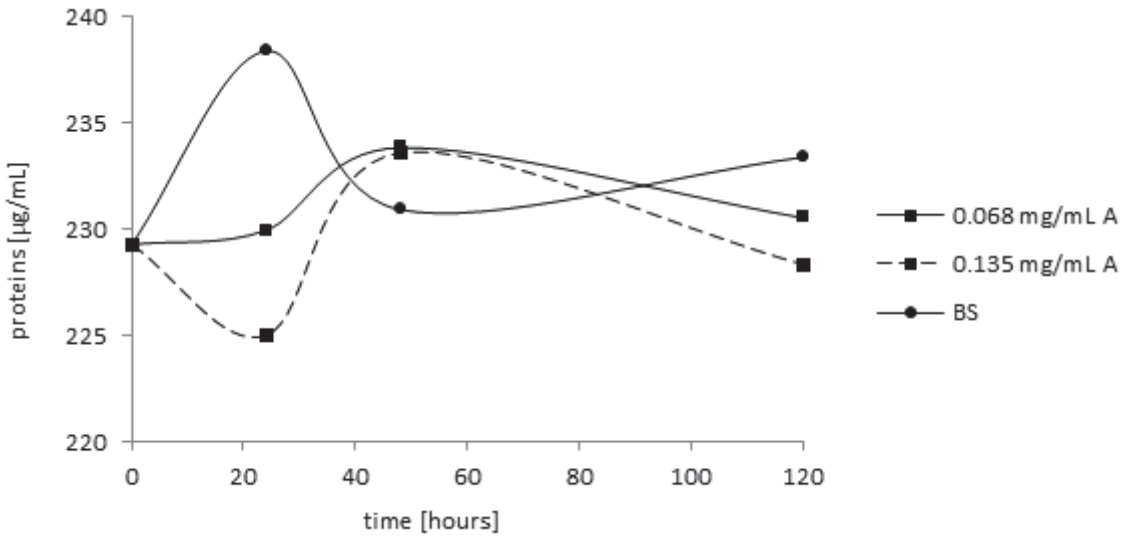


*Fig. 16 Time profile of concentration of proteins at shaking rate 160 rpm, cultivation medium inoculated with BS*

When comparing the results of *Fig. 15* and *16*, we can see that in the presence of higher concentration (0.135 mg/mL) of tested material, lower protein production and lower optical density are recorded.

This result indicates that increasing concentrations of tested material in the cultivation medium is associated with an increase of antimicrobial activity. This phenomenon correlates well with studies published by Liu *et al*. and Arias *et al*. [7, 52].

For all following experiments, the concentration of carbon nanomaterial about 0.135 mg/mL was chosen due to higher antimicrobial effect.

### 4.1.2 Screening of different incubation shaking rate

For this screening the cultivation was carried out by a submerged cultivation to achieve intensive aeration throughout the experiment and a homogeneous dispersion of the microorganism in a cultivation medium, which ensure the maximum utilization of nutrients and intensive reproduction of microbes.

The aeration should be important in production of proteins, because the essential role of oxygen in lipid metabolism and cell growth, for example, is well known [107].

Based on these findings, the shaking rate may influence the protein production; the influence on shaking rate on protein production by *BS* was examined in this work also.

For screening study two different shaking rates (160 rpm and 200 rpm) were examined. *BS* inoculum was cultivated at 30 °C in presence of tested carbon nanomaterial "A" for 120 hours at two above mentioned shaking rates. In four different cultivation periods the optical density and concentration of extracellular proteins were determined. As a control sample the NBG medium inoculated with *BS* without presence of the tested material was used.

The influence of shaking rate on bacterial growth is illustrated on *Fig. 17*. The significant growth of optical density during 24 hours of incubation was observed in presence of both

shaking rates and in control samples also. But generally the optical density reached higher values at shaking rate 200 rpm during the all examined period. These results correlate with finding reported by Alonso *et al*. [107].

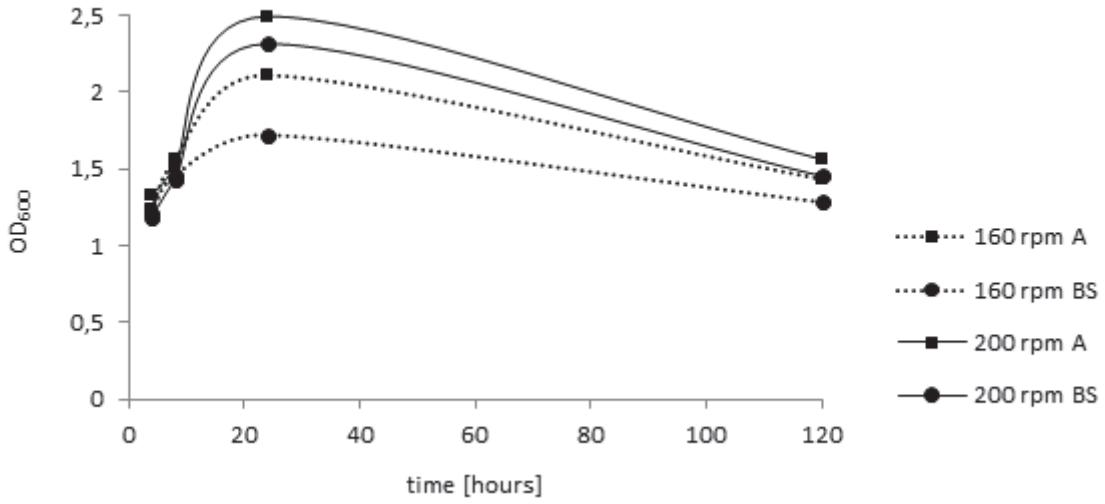


*Fig. 17 Time profile of optical density of BS in medium cultivated at different shaking rate (160 and 200 rpm) with and without tested carbon nanomaterial*

However, for lower shaking rate (160 rpm) more positive effect on extracellular proteins production in presence or without presence of tested material is noticeable (*Fig 18*).

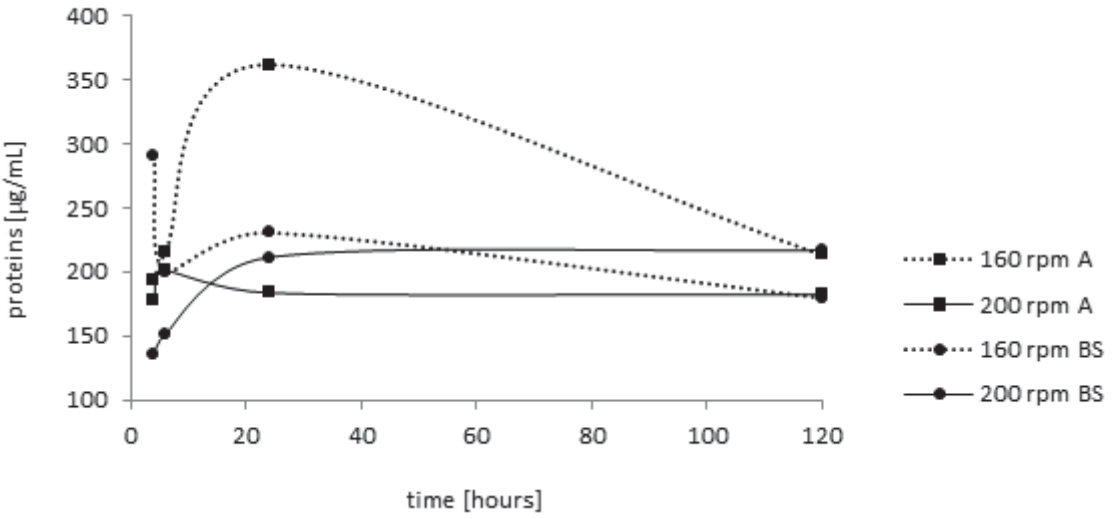


*Fig. 18 Time profile of concentration of extracellular proteins at shaking rate 160 and 200 rpm, cultivation medium inoculated with BS*

Based on the results of these screening studies it was concluded that all further measurements will be carried out with shaking rate of 160 rpm, because this shaking rate has more positive impact on protein production, as is on *Fig. 18* presented and second reason is that for the sample containing tested material and for the control sample, both shake at 160 rpm, a greater difference in optical density after 24 hours of incubation was observed than for samples shake at 200 rpm (*Fig. 17*).

These results cannot be compared with result in other studies, because the relationship between shaking rate and microbial growth in presence of carbon nanomaterials has not been published yet.

## 4.2 INFLUENCE OF CARBON NANOMATERIALS ON BEHAVIOUR OF SELECTED MICROORGANISMS

For the study of the effect of the nanoparticles on microorganism viability, various microorganisms, as bacteria, yeasts and fungi were used. Used microorganisms differ in cell wall composition, in resistance to foreign materials and in metabolism, for example [8, 108].

To characterize the antimicrobial properties of various materials, it is necessary to tested microorganism shows good growth in different environments. For this reason, the Gram-positive *Bacillus subtilis* was used as a model microorganism to evaluate the antimicrobial activity of different carbon-based nanomaterials. This bacterium is characterized by very good growth in different habitats. Biofilm formation and the production of EPS by *BS* were also documented in several studies [8, 74, 76, 85, 92, 105]. *BS* like Gram-positive bacterium due to a thick layer of peptidoglycan is much more susceptible to the effects of the nanoparticles than Gram-negative bacteria like as *E. coli* [109]. Because of mentioned susceptibility, *BS*

was used for testing of antimicrobial activity of several materials [6, 53, 108, 110].

The second used microorganism *Yarrowia lipolytica* represents yeast with good growth and also for this strain the biofilm formation was documented [111]. EPS production by *YL* was not documented yet. The yeast, generally, can survive in severely stressful conditions where is not possible for bacteria. The principal constituents of the yeasts cell wall are polysaccharides (chitin for example) with minor amounts of lipids and proteins [112]. This thick and complex cell wall can effectively block access of the nanoparticles to the cells, generally [109].

The inoculating methods described in points 3.2.1.2 (for *BS*) and 3.2.1.3 (for *YL*) were used for preparation of tested samples. In all experiments optimal cultivation temperatures (30 °C for *BS* and 28 °C for *YL*) were used. Other assays, such as optical density assay, growth kinetics, determination of total cell protein concentration and extracellular proteins concentration and determination of EPS follow the same procedure for both microorganisms. As a control sample the NBG medium inoculated with *BS* or basal medium containing Tween inoculated with *YL* without presence of the tested materials was used. These cultivation media with the best measured growth ability of microorganisms were chosen for mentioned microorganisms cultivation.

## 4.3 TIME-DEPENDENT EFFECT OF CARBON NANOMATERIALS ON MICROBIAL GROWTH AND EXTRACELLULAR PROTEIN PRODUCTION

For the determination of microbial growth, the spectrophotometric measurement (600 nm) of optical density was used. It is worth to mention that the spectrophotometric measurement provides indirect information about bacterial growth, which could be influenced also by presence of dead cells. The optical density was determined in inoculated medium after 4, 8, 24 and 120 hours of incubation (30 °C for *Bacillus subtilis* and 28 °C for *Yarrowia lipolytica*) at shaking rate 160 rpm in presence of three types (“A”, “B” and “C”) of tested carbon nanomaterials (0.135 mg/mL).

Determination of production of total cell proteins and extracellular proteins was performed spectrophotometrically using default program which counts amount of protein per volume unit on Spectrophotometer SPEKOL (Germany, UV-VIS method) in inoculated medium after 4, 8, 24 and 120 hours.

### 4.3.1 Bacterial growth

Generally, the bacterial growth curve can be divided into four parts. During the initial lag phase the increase of numbers of cells is very slow. The cells are enzymatically preparing for growth in new medium or under new conditions and synthesis of RNA, enzymes and other molecules occurs [113]. Initial lag phase is not evident from *Fig. 19* since it occurs in a shorter cultivation period than 4 hours. The exponential phase like a period characterized by cell doubling [113] is very good visible from *Fig. 19* in presence of all tested materials and in control sample as well. The following stationary phase (24 hours – 120 hours on *Fig. 19*) occurs often due to a growth-limiting factor such as the enervation of essential nutrients or inhibitory metabolites build up. The death phase of the bacterial growth cycle, when bacteria

depleted nutrients and die [113], would be reflected as decrease of optical density. However in our case the death phase was not detected, because occurs after longer cultivation period than 120 hours. Finally we can report, that bacterial growth of *BS* goes in the same course in presence of all three types of tested material and in control sample as well.

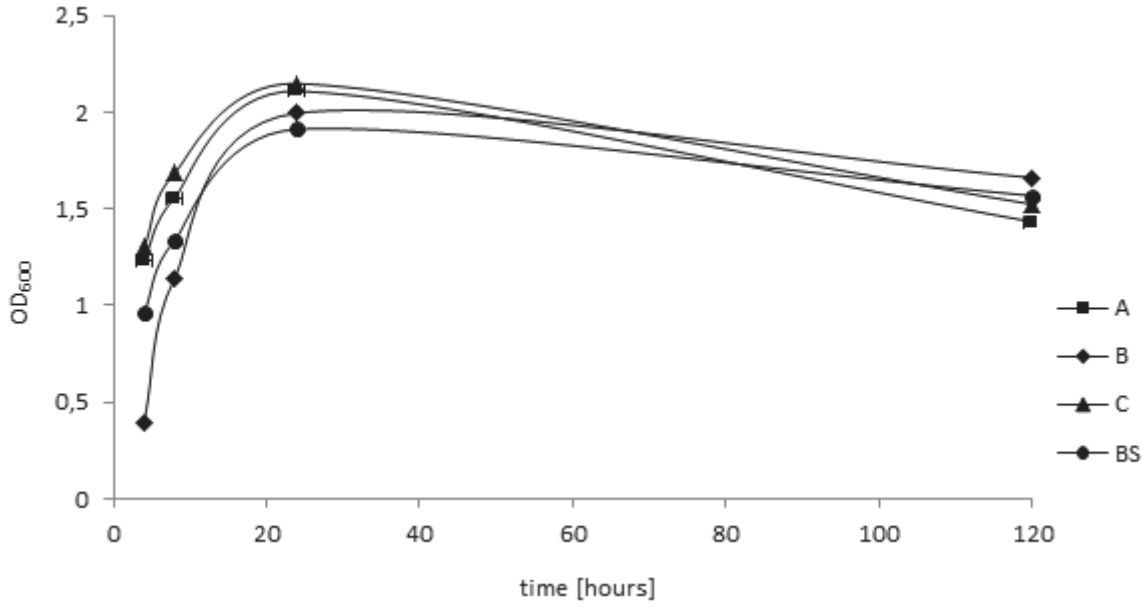


*Fig. 19 Time profile of BS bacterial growth expressed in optical density during cultivation at shaking rate 160 rpm*

### 4.3.2 Yeast growth

The first phase – lag phase is very good visible during first 8 hours of cultivation period followed by exponential phase (*Fig. 20*) in all tested samples, as before. The fact that in *YL* growth lag phase is good observed in comparison with *BS*, could be due to different physiology of microorganisms. Stationary phase is not good visible, because of ending cultivation after 120 hours. There are no significant differences between mode of growth of *YL* both in the presence and in the absence of tested carbon nanomaterials *(Fig. 20)*.

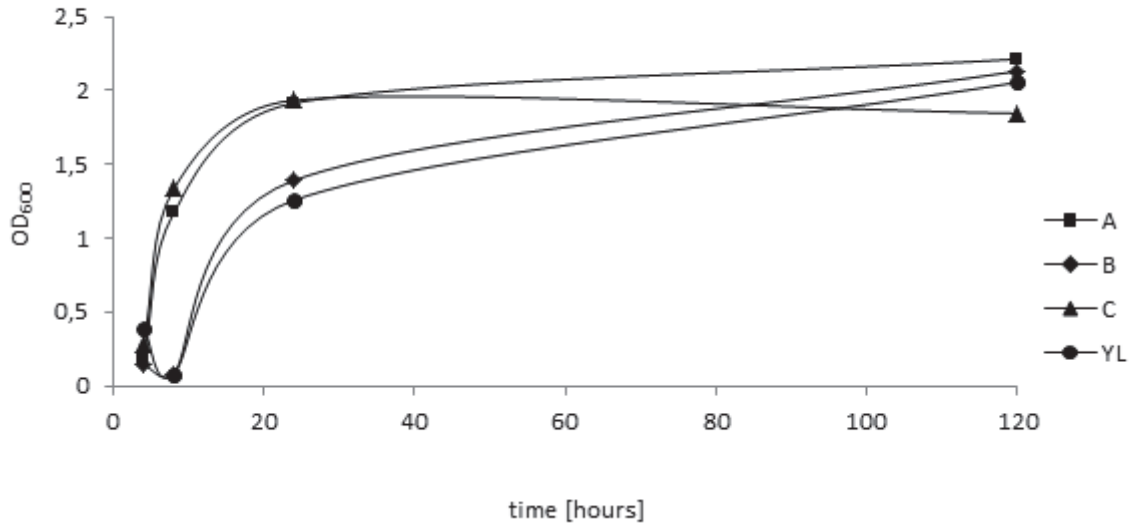


*Fig. 20 Time profile of YL yeast growth expressed in optical density during cultivation at shaking rate 160 rpm*

Summary we should note the growth pattern of *BS* and *YL* in presence of tested carbon nanomaterials does not show a significant differences compared with mode of growth of *BS* and *YL* in absence of tested material; thus the influence of tested materials on growing rates, determined on the basis of optical density, of mentioned microorganisms is not very significant, how could be expect. Optical density is widely used method for the basic monitoring studies [8, 114].

### 4.3.3 Concentrations of total cell proteins and extracellular proteins during cultivation experiments

The concentration of total cell proteins gives us overview of cell growth under different conditions and in different times of incubation. In contrast, the concentration of extracellular proteins shows the production of substances into the environment, which may be affected by the presence of foreign materials, like tested carbon nanomaterials in this work.

To better determination the effect of tested carbon nanomaterials on the viability and metabolism of *BS* and *YL* cells, concentrations of total cell proteins and extracellular proteins during cultivation experiments were monitored.

The noticeable increase of total cell proteins concentration of *YL* during cultivation period can be observed in presence of all tested material and in control as well (*Fig. 23*). In the *BS*-inoculated medium this trend cannot be observed, the total cell proteins concentrations (in presence of tested materials and in control sample) increase or decrease slightly during 24 hours and then is constant until the end of cultivation period (*Fig. 21*).

The same trend is possible to see in monitoring of extracellular proteins concentration in *BS*-inoculated medium (*Fig 22*). Compared with this, in the *YL*-inoculated medium, the

increase of extracellular proteins concentration after 6 hours of cultivation is striking. Followed by, marked decrease after 24 hours of cultivation. Afterwards we can see slow increase until the 120 hours (*Fig. 24*).

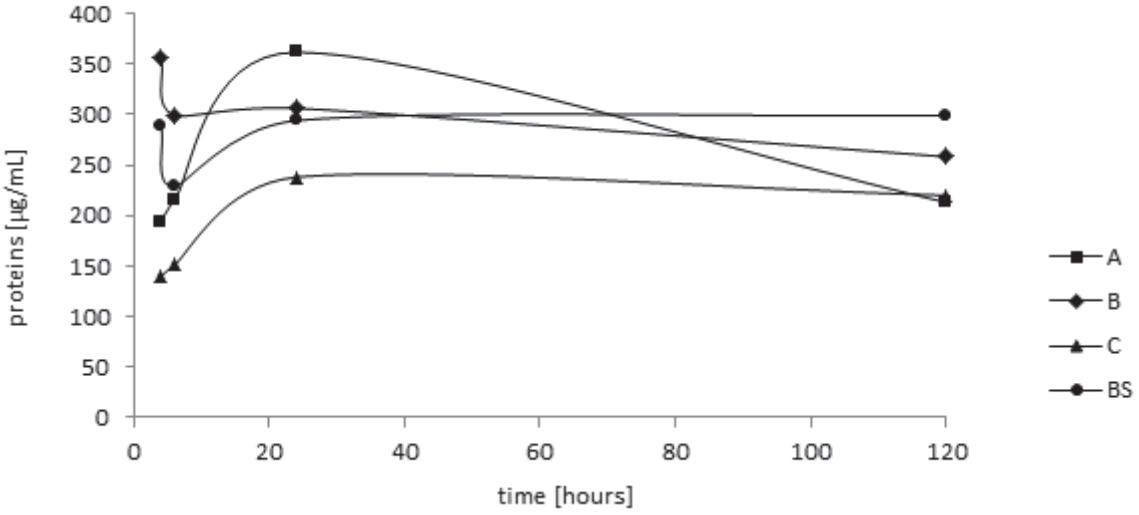


*Fig. 21 Time profile of concentration of total cell proteins at shaking rate 160 rpm, cultivation medium inoculated with BS*

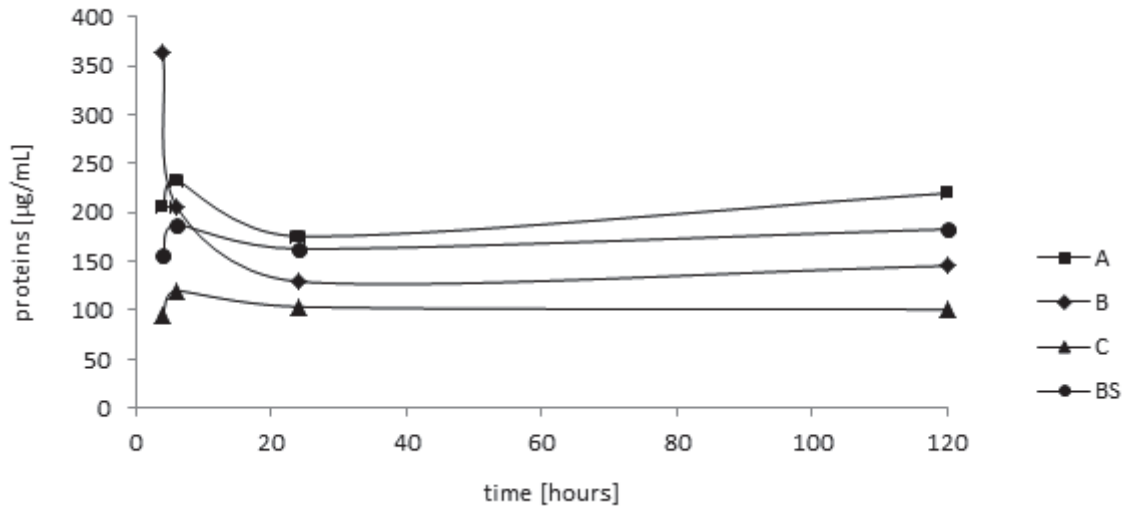


*Fig. 22 Time profile of concentration of extracellular proteins at shaking rate 160 rpm, cultivation medium inoculated with BS*

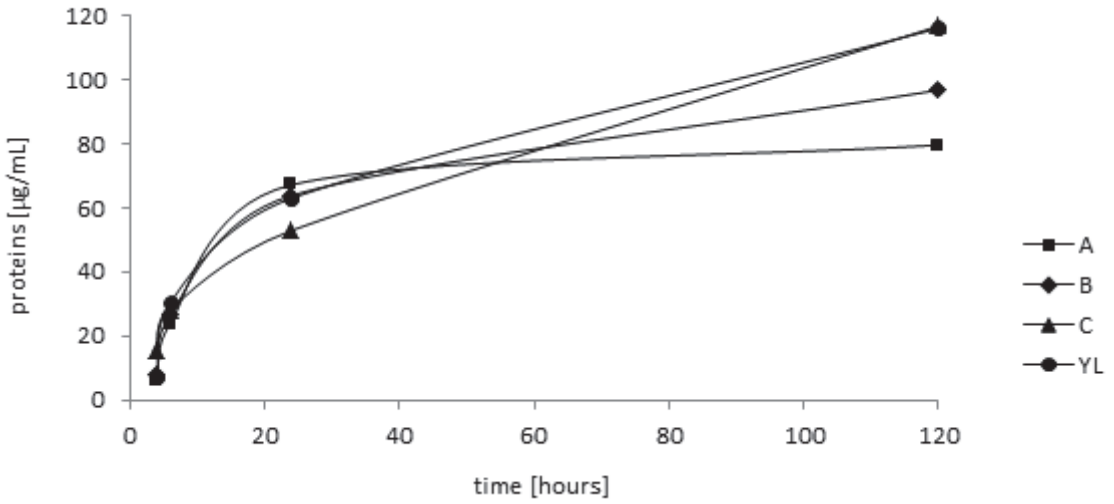


*Fig. 23 Time profile of concentration of total cell proteins at shaking rate 160 rpm, cultivation medium inoculated with YL*

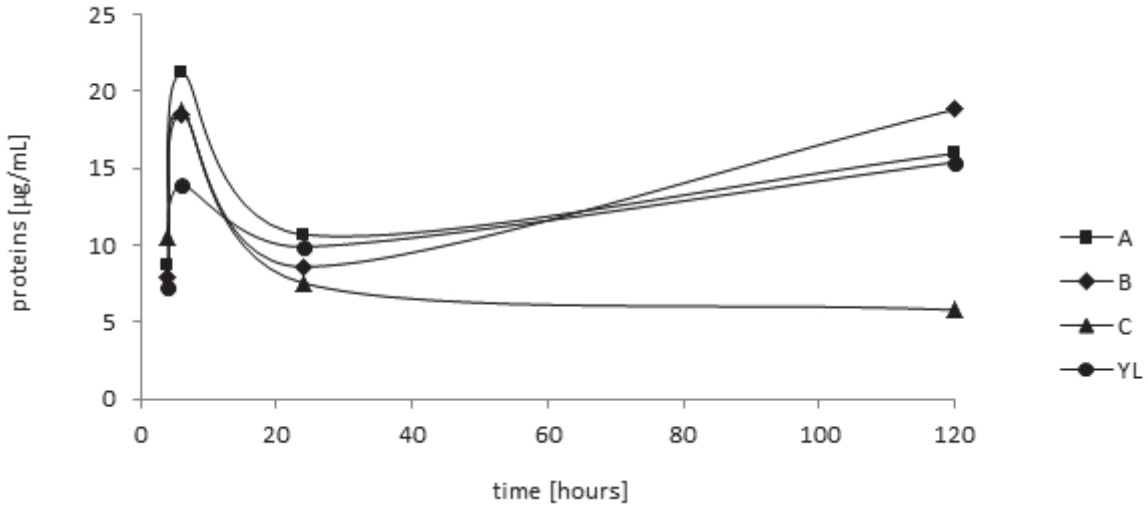


*Fig. 24 Time profile of concentration of extracellular proteins at shaking rate 160 rpm, cultivation medium inoculated with YL*

With focus on yeast *YL*, presence of all types of tested carbon nanomaterials has a positive impact on yeast growth (*Fig. 23*); but in contrast the protein production by *YL* is not significantly affected by the carbon nanomaterials presence; only the slightly increase until the end of cultivation period is possible to observe (*Fig. 24*). The increasing trend observed on *Fig. 23* is possible to compare with increase of optical density of *YL*-inoculated medium (*Fig. 20*). Next, it is possible to note, on *Fig. 20* the exponential phase is observed in time range 24 – 120 hours, in the same period the concentration of extracellular proteins was measured (*Fig. 24*); the slightly increase of concentration of these proteins could be due to higher production

of enzymes during the exponential phase of growth; such as very common for yeast metabolism [113].

With focus on *BS* cells, there is no significant impact of tested materials on concentrations of total cell proteins and extracellular proteins (*Fig. 21* and *22*).

Because of different yeast and bacterial metabolisms the measured concentrations of *YL* proteins are overall lower during the all incubation period than the concentration of *BS* proteins.

## 4.4 ANTIMICROBIAL ACTIVITY

Firstly is important to say, there is no consensus in the literature regarding biocompatibility and antimicrobial activity of graphene materials in general. Newly, Ruiz *et al*. displayed that bacteria grew faster and to a higher optical density when material GO was added to a bacterial culture in concentration 25 μg/mL than cultures without GO [8]. In contrast Liu *et al*. detected strong antibacterial activity of four kinds of graphene-based materials (Gt, GtO, GO, and rGO) in concentration 40 μg/mL [7]. Liu *et al.* also reported time-dependent antimicrobial activities of GO and rGO materials expressed in loss of viability (%) of *E. coli* cells [7]. Similar observation was presented by Gurunathan *et al*., which reported decrease of optical density in presence of GO and rGO (75 μg/mL) materials during incubation period 15 hours [22].

In this thesis *B.subtilis* and *Y.lipolytica* were used to evaluate potential antibacterial activity of three different types of carbon nanomaterials.

*B.subtilis* cells (30 °C) and *Y.lipolytica* cells (28 °C) were incubated with the same concentration (0.135 mg/mL) of tested materials dispersions (“A”, “B” and “C”) in cultivation medium at 160 rpm for 144 hours. At regular intervals, samples were taken and antimicrobial activity of microbial cells was determined by colony counting method (for *BS*) and counting method of live cells in Bürker counting chamber (for *YL*) described in the method section.

Antimicrobial activity of tested materials is expressed like a loss of viability of *BS* and *YL* cells. For this experiments control samples inoculated with *BS* or *YL* with no tested material were used as samples with 100 % of viability; with no loss of viability respectively.

* On following figures negative values indicate positive growth of cells.

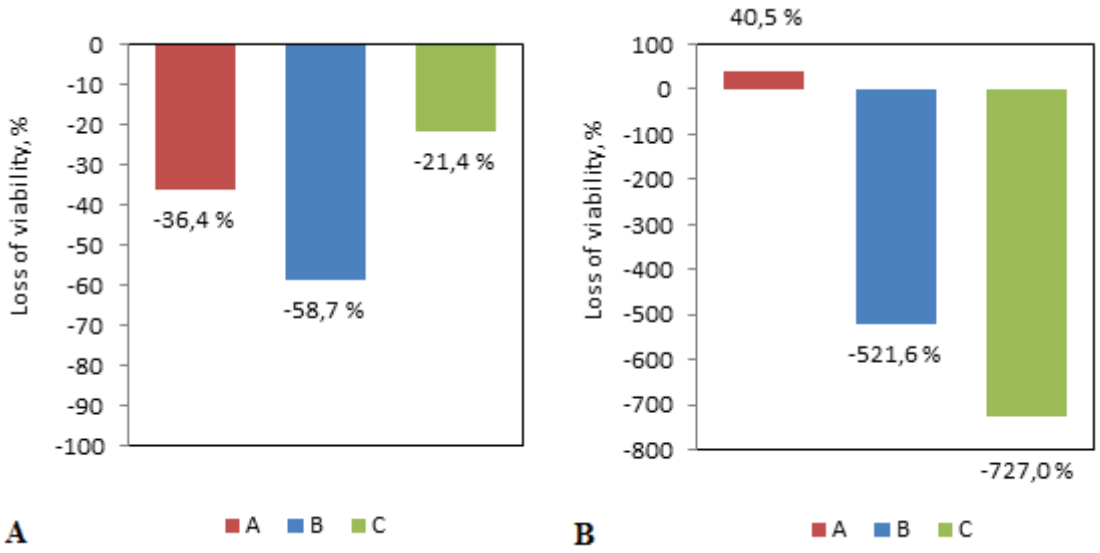


*Fig. 25 Loss of viability of YL (A) and BS (B) cells after 6 hours of cultivations*

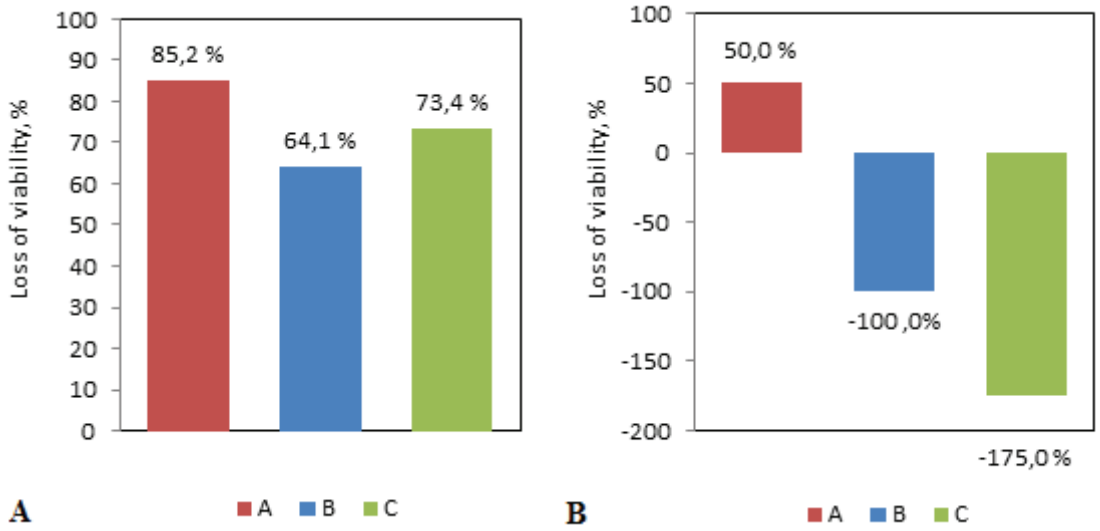


*Fig. 26 Loss of viability of YL (A) and BS (B) cells after 48 hours of cultivations*

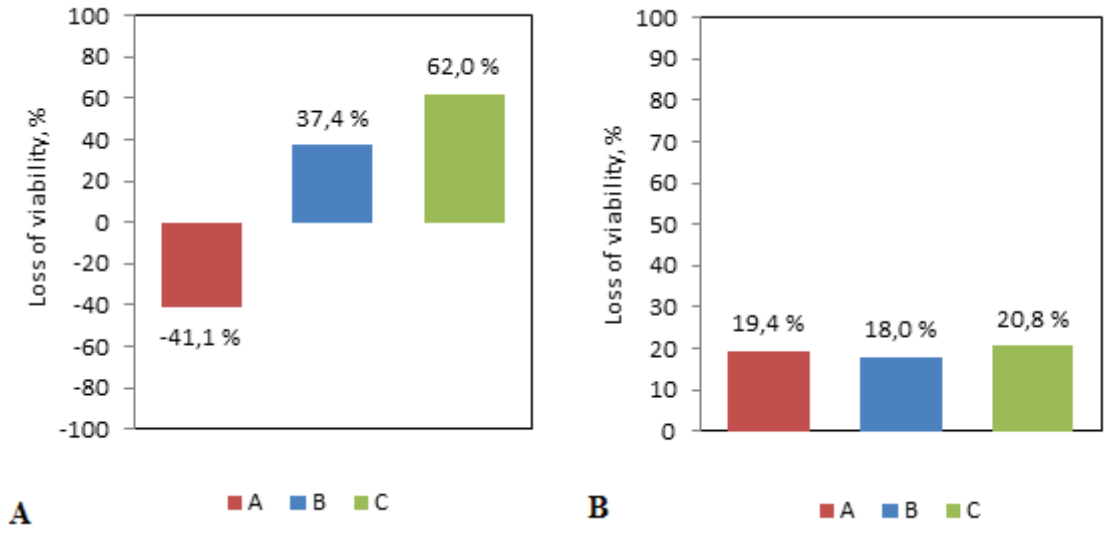


*Fig. 27 Loss of viability of YL (A) and BS (B) cells after 144 hours of cultivations*

From the results of loss of viability of microbial cells can be seen that at the beginning of incubation (during first 6 hours) the presence of tested carbon nanomaterials stimulates growth of *YL* cells (*Fig. 25 A*). This effect is more pronounced in the presence of the "A" and "B" materials in comparison with material "C". This could be related to the fact, that those carbon materials "A" and "B" have smaller sizes of particles than material "C",which is prepared by thermal reduction and also exhibits a lower degree of oxidation.

Tested nanomaterials exhibit the antimicrobial effect after a longer incubation time – 48 hours with the cell inactivation percentages 63-85 % (*Fig. 26 A*). The materials "B" and "C" showed cytotoxic effect for the rest of experiment (until 144 hours) with comparison to materials "A", which begins to again support growth of yeast after 144 hours (*Fig. 27 A*).

*BS* cells were incubated with the same nanomaterials and the growth of *BS* was stimulated in presence of tested carbon nanomaterials "B" and "C" during 48 hours of cultivation period as well, contrary to material "A" which showed moderate antimicrobial activity with loss of viability about 40-50 % (*Fig. 25 B* and *26 B*). All tested nanomaterials exhibited slight cytotoxicity after longer cultivation period – 144 hours (*Fig. 27 B*).

Monitored cytotoxic effect is time dependent and also microbial strains used play an important role. On the basis of the presented results, we conclude that for evaluation of antimicrobial effect of carbon nanomaterials, synergistic action of several factors such as the degree of oxidation of tested nanomaterials, the different behavior of materials in the cultivation medium due to possibility of the aggregation of graphene nanosheets, which is supported as important antibacterial mechanism, must take into account [7]. There are still lots of questions about antimicrobial effect of carbon nanoparticles and it is expected that this issue will be further studied and explored.

## 4.5 DETERMINATION OF EPS

Extracellular polymeric substances (EPS) are one of the most important components in microbial biofilm, which serve to reinforce its structural integrity and confer increased antimicrobial resistance [74, 114]. The EPS determine the immediate life conditions of cells living in biofilm microenvironment [83, 95]. In summary, we assume that the presence of EPS indicates the formation of biofilm and their role can also be associated with cell protection against nanoparticles toxicity [115]. For these reasons, we monitored the level of EPS production of studied microorganisms.

In EPS three main components (proteins, reducing substances and exopolysaccharides) were monitored. For these determinations the methods describe in 3.2.5, 3.2.6 and 3.2.7 were used.

EPS were determined during short time period – until 24 hours, due to encourage growth in this time period; based on the results in section 4.4. High productivity of EPS molecules was observed during exponential phase of cell growth, which can be connected with significant cell growth and in stationary phase decrease in EPS production was monitored.

EPS were determined by ethanol precipitation method. As mentioned by several authors, yields of EPS extracted from biofilm depend on the extraction method. Precipitation by ethanol was selected in this study, because this precipitation process removes only the biopolymer (exopolysachcharides and proteins) and not corresponding monomers such as glucose present in the fermentation medium [116].

*B.subtilis* cells (30 °C) and *Y.lipolytica* cells (28 °C) were incubated with the same concentration (0.135 mg/mL) of tested materials dispersions (“A”, “B” and “C”) in cultivation medium at 160 rpm for 24 hours. At regular intervals, samples were taken and concentrations of proteins, reducing substances and total sugars were measured.

### 4.5.1 Determination of concentration of proteins

First of all, the concentration of produced proteins and polypeptides which have the potential to induce aggregation of particles which are important for biofilm formation [115] and bound the cells in biofilm together was determined [84]. Proteins also play essential role with other extracellular matrices, like polysaccharides, for example, in the establishment and maintenance of biofilm structure together [74].

The significant increase of concentration of proteins produced by *BS* was determined during the all cultivation period (*Fig. 28*). ) and is more pronounced in the presence of tested materials (all three types) as compared with control sample. In general, in the presence of tested carbon nanomaterials *BS* produce a larger amount of extracellular proteins than in the absence of these materials. It could be assumed that the increased production of proteins is response to the presence of carbon nanoparticles and could be a defense mechanism before the cytotoxicity of the nanoparticles. This observation could be also related to the fact that the growth of *BS* cells was stimulated in presence of materials “B” and “C” during 48 hours of cultivations and thus an increased amount of EPS in the environment promotes cell growth (*Fig. 25 B* and *26 B*, section 4.4.).

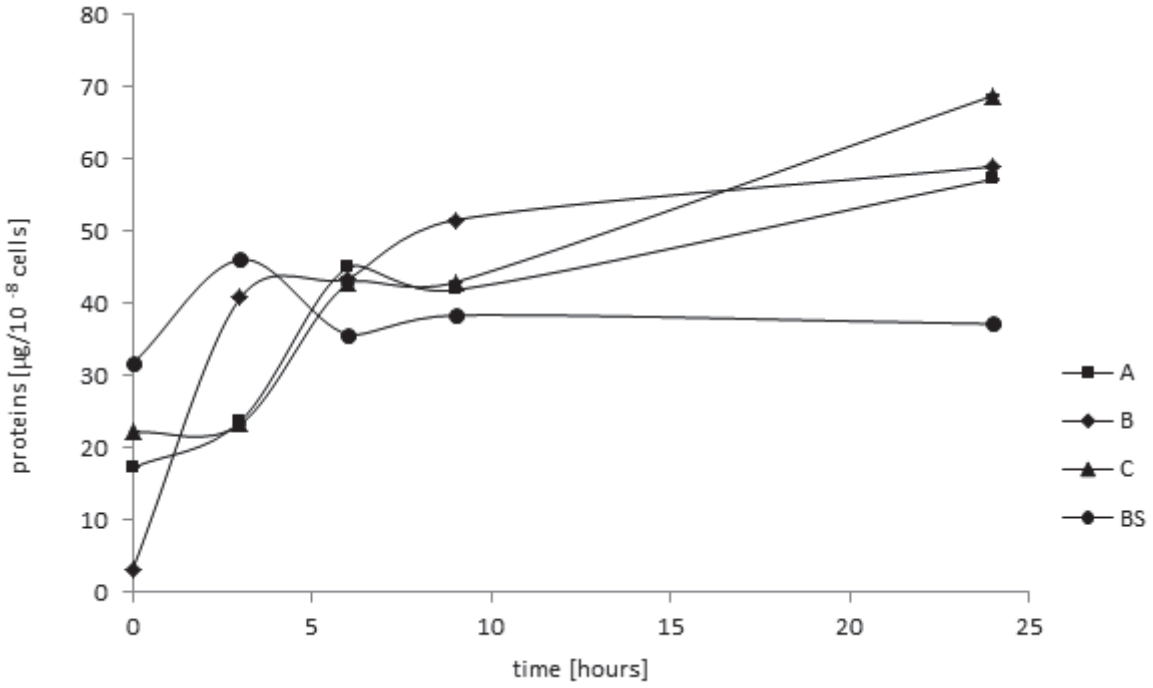


*Fig. 28 Time profile of concentration of proteins in EPS at shaking rate 160 rpm, cultivation medium inoculated with BS*

In the *YL*-inoculated samples the significant protein production was not detected (*Fig. 29*). Only in the presence of tested material "B" the concentration of proteins increase until the end of the examined period (24 hours).

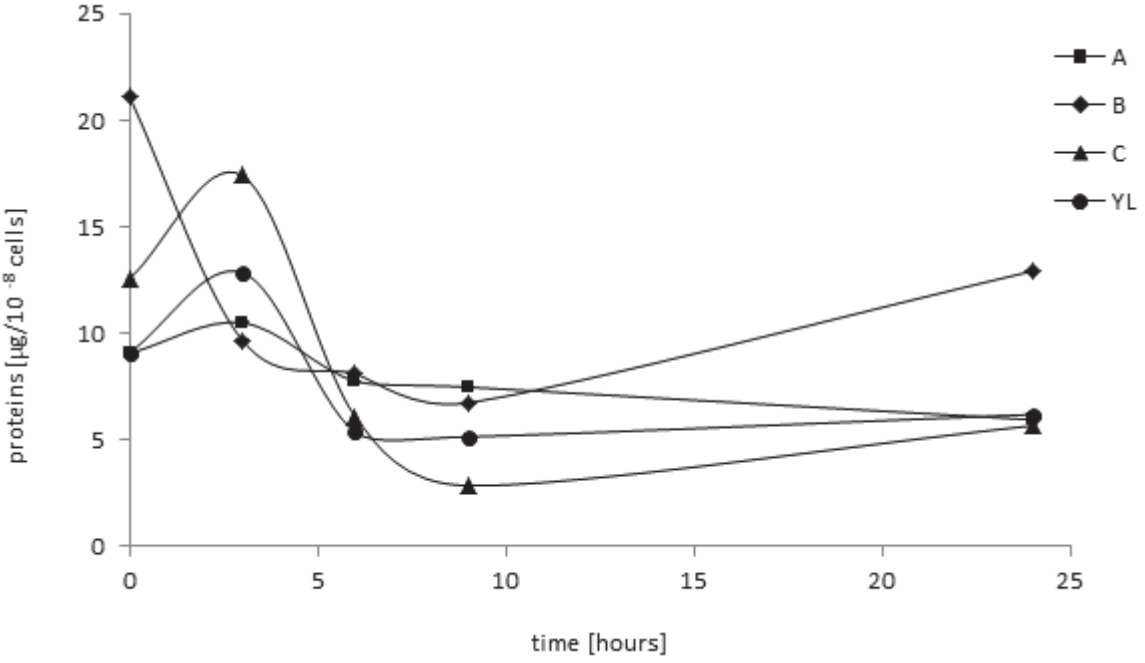


*Fig. 29 Time profile of concentration of proteins in EPS at shaking rate 160 rpm, cultivation medium inoculated with YL*

In summary can be noted that bacterium *BS* produce much more proteins than yeast *YL* during the examined time period. In *YL*-inoculated medium the maximum concentration of proteins is 21 $\mu g/10^{-8}$ cells, while in *BS*-inoculated medium it is almost 70 $\mu g/10^{-8}$ cells. Therefore *BS* responds to the presence of foreign carbon nanoparticles by increasing production of proteins more markedly.

### 4.5.2 Determination of concentration of reducing substances

Sugars (for example: glucose, galactose, fructose, maltose, lactose, and pentose) are characterized as reducing substances based on their ability to reduce cupric ions to cuprous ions [106]. Qurashi *et al*. reported that reducing sugars are one of the major components of EPS produced by bacterium that are increased in the presence of higher salt stress and increases the biofilm stability of bacterial cells [117].

We can see sharp increase of concentration of reducing substances in *BS*-inoculated medium during first 3 hours of cultivation (*Fig. 30*). This may be due to the increase of reducing substances production as response to the carbon nanoparticles presence. Afterwards the decrease of reducing substances concentration was observed until the end of cultivation period. From these results, it is not possible to record that the reducing substances production by *BS* cells is the response on the presence of foreign nanoparticles during the all exposition time.

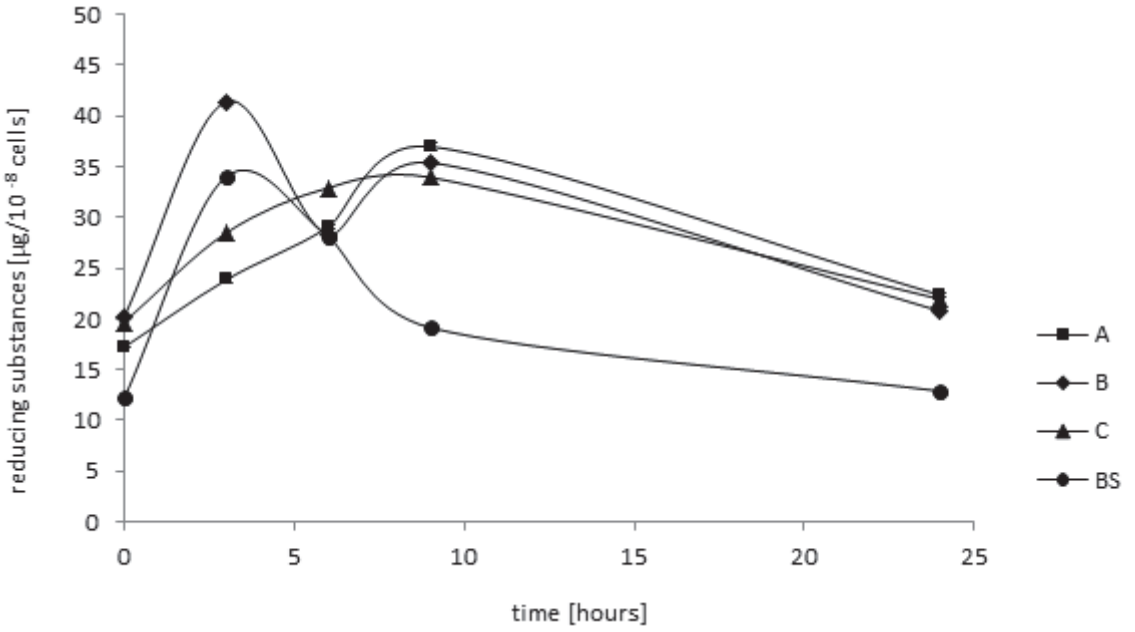


*Fig. 30 Time profile of concentration of reducing substances in EPS at shaking rate 160 rpm, cultivation medium inoculated with BS*

In tested samples inoculated with *YL* cells (*Fig. 31*) the course of content of reducing substances is similar to samples with *BS* cells.

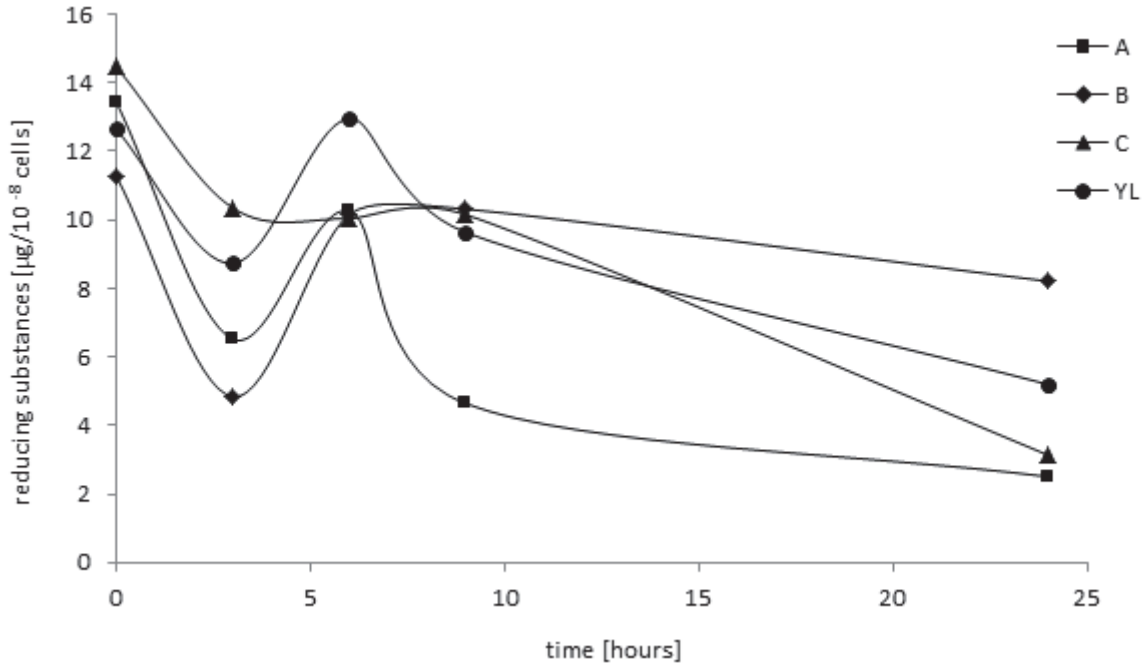


*Fig. 31 Time profile of concentration of reducing substances in EPS at shaking rate 160 rpm, cultivation medium inoculated with YL*

### 4.5.3 Determination of concentration of total exopolysachcarides

Exopolysaccharides (total sugars) are other major component of EPS. Exopolysacharides secreted by bacteria could play an important role in controlling the toxicity of nanoparticles in the environment and acts as a physical barrier against particles toxicity [115]. As is already mentioned in the section 4.5.1., exopolysaccharides play also essential role in the establishment and maintenance of biofilm structure together with proteins and polypeptides [74].

Therefore, in this work determination of the concentration of exopolysaccharides produced by selected microorganisms is performed during cultivation.

The amount of exopolysaccharides secreted by *BS* increases in presence of tested materials during the cultivation period and the amount is higher than in control sample without tested materials in the end of cultivation period (*Fig. 32*). It could be considered that the increased production of total sugars is the reaction of *BS* cells to presence of carbon nanoparticles.

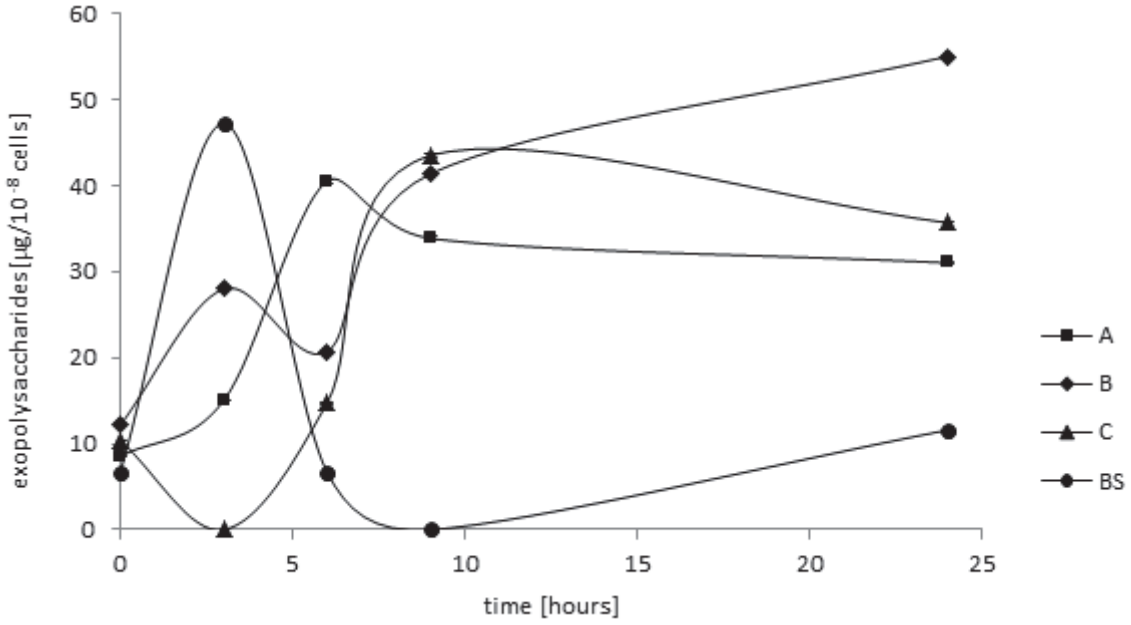


*Fig. 32 Time profile of concentration of total sugars in EPS at shaking rate 160 rpm, cultivation medium inoculated with BS*

The amount of exopolysaccharides secreted by *YL* increases in the presence of tested materials in the beginning of the cultivation period and decreases until the end of cultivation period (*Fig. 33*).

In *BS*-inoculated medium is possible to detect higher amount of exopolysaccharides than in *YL*-inoculated medium: maximum exopolysaccharides concentration of 28 $\mu g/10^{-8}$ cells in *YL*-inoculated medium in comparison with more than 50 $\mu g/10^{-8}$ cells in *BS*-inoculated medium. Therefore *BS* responds to the presence of foreign carbon nanoparticles by increasing production of proteins but also of exopolysaccharides.

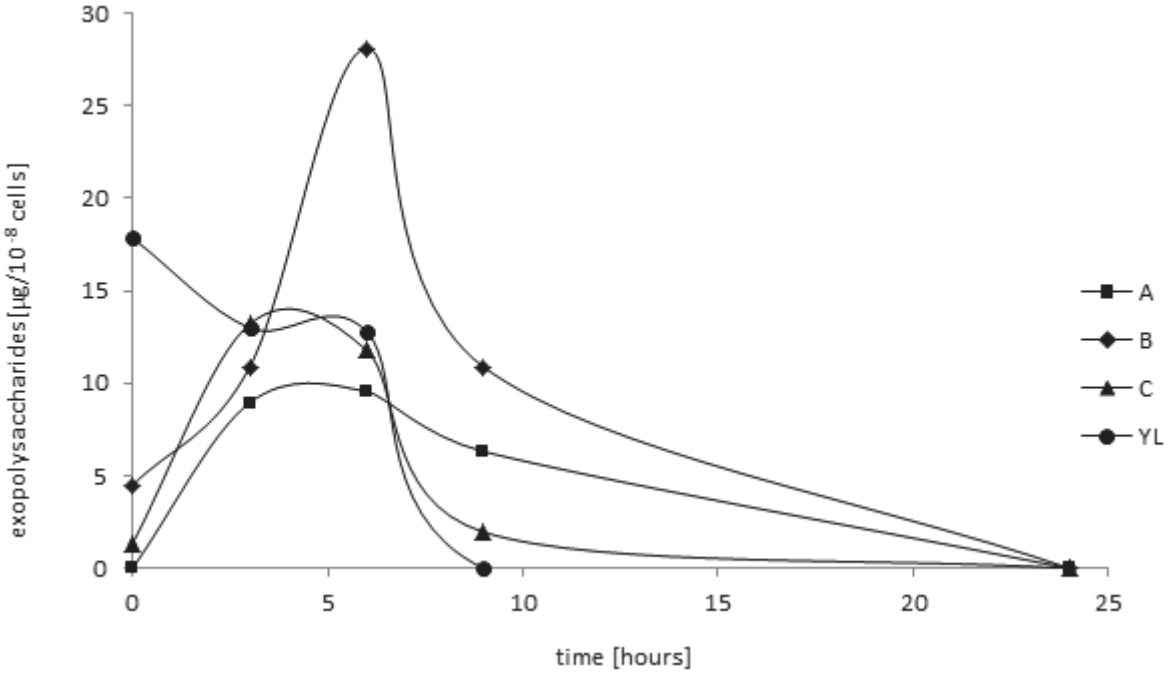


*Fig. 33 Time profile of concentration of total sugars in EPS at shaking rate 160 rpm, cultivation medium inoculated with YL*

Based on the results obtained by measuring of EPS production, we assume the higher biofilm formation in *BS*-inoculated medium in comparison with *YL*-inoculated medium. Bacterium *BS* produces higher amount of proteins and exopolysaccharides than yeast *YL* (*Fig. 28, 29, 32* and *33*). This finding correlate with information, that *Bacillus subtilis* strains produce a wide variety of EPS, as is published by Marvasi *et al*. [105]. Due to a massive biofilm formation and EPS production by *BS*, this bacterium could be better protected from the action of carbon nanomaterials, tested in this work, than tested yeast. This finding could be supported by the results reported in section 4.4.; *YL* cells show appreciable higher loss of viability after a longer incubation period in presence of tested carbon materials than *BS* cells.

The results presented in this thesis cannot be compared with result in other studies, because the relationship between EPS production and antimicrobial activity of nanomaterials has not been published yet.

# 5 CONCLUSIONS

The aim of this thesis was to provide a screening study about antimicrobial effect of carbon-based fillers on viability and extracellular polymeric substances production of bacterium *Bacillus subtilis* (CCM 1999) and yeast *Yarrowia lipolytica* (CCY 29-26-52).

It could be presented, that *Bacillus subtilis* and *Yarrowia lipolytica* cells react in other way to presence of carbon nanomaterials, which is reasonable due to their different metabolisms and cell wall composition and thus their different resistance to the cytotoxicity of these particles.

It could be assumed that *Yarrowia lipolytica* cells show better resistance than *Bacillus subtilis* cells to tested materials, based on general knowledge about cell wall compositions. However, the results obtained it this work show, that the cell wall composition does not have such an influence to resistance to carbon nanoparticles, one would expect. It should be more taken in consideration, that the biofilm formation and extracellular polymeric substances (EPS) production could enhance the resistance of mentioned microorganisms. This suggestion is strongly supported, based on following results: *Bacillus subtilis* cells are better protected due to massive biofilm creation and EPS production than *Yarrowia lipolytica* cells, which produce fewer amounts of these substances.

The remaining question is, if the elevated production of EPS is caused by reaction to foreign particles, it means self-protection or by the biofilm creation. It should be considered this issue for further research.

The EPS production related with the presence of carbon nanoparticles is really interesting topic since it has not been published yet.

The size of carbon nanoparticles, degree of oxidation and level of material homogenization in cultivation medium are another three factors which could have an influence to particles toxicity. Due to this prerequisite three different particles (labeled as “A”, “B” and “C”) were examined; the material “C” has the biggest size of particles with the lowest degree of oxidation.

It should be necessary to do more complex and more precise tests, such as TEM, for more precise description of nanoparticle effects on mentioned microorganisms and on protective mechanism which means influence of biofilm creation and EPS substances production.

This work is primarily created as a screening study, which should point on interesting factors in this issue and it is expected that this issue will be further studied and explored.

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# 7 LIST OF ABREVIATIONS

| | |
|---|---|
| 2D | Two-dimensional |
| BS | *Bacillus subtilis* |
| BSA | Bowine serum albumin |
| BM | Basal medium |
| CCM | Czech Collection of Microorganisms |
| CFU | Colony forming units |
| CNMs | Carbon nanomaterials |
| CNTs | Carbon nanotubes |
| DNA | Deoxyribonucleic acid |
| e-DNA | Extracellular DNA |
| EPS | Exopolymeric substances |
| GFNs | Graphene-Family Nanomaterials |
| GO | Graphene oxide |
| Gt | Graphite |
| GtO | Graphite oxide |
| IARC | International Agency for Research on Cancer |
| LB-EPS | Soluble EPS (slime polymers) |
| MPA | Meet-Peptone Agar |
| MWCNTs | Multiwall carbon nanotubes |
| NBG | Nutrient Broth + Glucose |
| OD | Optical density |
| rGO | Reduced graphene oxide |
| RNA | Ribonucleic acid |
| ROS | Reaction oxygen species |
| rpm | Revolutions per minute |
| SEM | Scanning electron microscope |
| SMP | Soluble cellular components |
| SWCNTs | Single walled carbon nanotubes |
| TB-EPS | Bound tightly EPS |
| YL | *Yarrowia lipolytica* |